UNIVERSITEIT GENT

FACULTEIT WETENSCHAPPEN

The role of membrane vesicle secretion in *Stenotrophomonas maltophilia* antibiotic resistance

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2015

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Dissertation submitted in fulfilment of the requirements for the degree of Doctor (Ph.D.) in Sciences, Biochemistry

The research reported in this thesis was performed at the
Laboratory for Protein Biochemistry and Biomolecular Engineering (L-ProBE)
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This work was supported by IWT-Vlaanderen

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Acknowledgements

First of all I want to express my gratitude to Prof. Bart Devreese. I started as a master student in his lab, then applied for a PhD scholarschip, and eventually ended up as PhD student for a period of four years. His continuous support throughout this period was of great value. I also want to thank Laurence for introducing me into the lab and sharing your proteomics wisdom with me.

Off course I want to thank the whole L-PROBE team (past and present). Whether it was working, not working, talking, laughing, arguing, complaining, cleaning, eating or drinking, it's nice to do it with great colleagues. And the daily commute to work was (for a couple of years) considerably more pleasant thanks to ex-colleague Griet. Other commuters who enjoy a silent trip to work might think differently (just kidding). A special thanks to Gonzalez for his support with rogue computers and crazy LC-MS systems, to Wouter for his excellent microscopy work, and to Stephan for quantifying billions of vesicles. I also want to thank former master students Sofie, Berten and Jolien for assisting me during my PhD years.

Finally I want to thank my parents for their unconditional trust and support. I simply could not have done it without you. Also thanks to my brothers, all my friends, and my whole family for many good times.

Most importantly, I want to thank Wendy for always being there for me, and for giving me a wonderful son along the way.

English summary

Stenotrophomonas maltophilia is an emerging multidrug resistant nosocomial pathogen, mostly associated with chronic pulmonary infections and medical device-associated infections. Treatment of *S. maltophilia* infections is compromised due to its many mechanisms of resistance against different families of antibiotics such as β-lactams, aminoglycosides, quinolones, tetracyclines, macrolides and sulfonamides.

Former proteomic studies on the cellular response of the clinical *S. maltophilia* strain 44/98 showed an increase in expression of two outer membrane Ax21 homologues after treatment with the β-lactam imipenem. Ax21 was previously shown to be involved in virulence and biofilm formation, and to be secreted in association with outer membrane vesicles (OMVs). Therefore, we characterized the imipenem-induced OMVs to assess their role in β-lactam resistance. OMV proteomics revealed a diverse protein cargo, with mainly outer membrane and periplasmic proteins, but also inner membrane and cytoplasmic proteins. Interestingly, the L1-metallo-β-lactamase and the L2-serine-β-lactamase were also identified as part of the OMV proteome. Next to imipenem, we investigated the effect of diffusible signal factor (DSF) quorum sensing molecules on OMV production. In the closely related plant pathogen *Xylella fastidiosa*, a DSF synthase knock out strain produced more OMVs, pointing to an inhibitory role of the DSF system. In contrast, a stimulatory effect was observed in *S. maltophilia* after exposure to its own DSF, cis-Δ2-11-methyl-dodecenoic acid. Moreover, exposure to the *Burkholderia cenocepacia* DSF cis-Δ2-dodecenoic acid also induces an increase in OMV production. Imipenem, cis-Δ2-11-methyl-dodecenoic acid and cis-Δ2-dodecenoic acid all lead to abundant OMV-associated secretion of the two Ax21 homologues, but the expression of both homologues seems to be differentially regulated. Unfortunately, the actual role of Ax21 is still unknown and presents an interesting topic for further research.

Both chromosomal encoded β-lactamases were identified as part of the OMV cargo. Next, the intra- and interspecies impact of OMVs was examined on β-lactam tolerance. A nitrocefin β-

lactamase assay on intact OMVs indicated an equal distribution between L1 and L2 activity, while the cellular activity is predominantly achieved by L1. The clinically relevant β-lactams imipenem, amoxicillin and ticarcillin were also shown to be degraded by intact β-lactamase-carrying OMVs, but all with different efficiency. *S. maltophilia* often thrives in polymicrobial biofilm communities with species like *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*, especially in cystic fibrosis-associated chronic lung disease. Therefore, the ability of *S. maltophilia* β-lactamase-packed OMVs to confer intra- and interspecies protection against β-lactams was investigated. The OMVs not only increase the β-lactam tolerance of *S. maltophilia* cultures, but also have drastic effects on the tolerance of the less resistant *P. aeruginosa* and *B. cenocepacia* cultures. These findings suggest the possibility of *S. maltophilia* OMVs to aid in intra- and interspecies protection against β-lactam antibiotics. Furthermore, biofilm mode of growth also aids in antibiotic protection. In several species, OMVs were shown to be important for biofilm initiation and maintenance. Here, β-lactam-induced vesicles from *S. maltophilia* were proven to inhibit its transition to biofilm, most likely through blocking of available surfaces. Strikingly, the opposite effect was observed when these OMVs were administered to *P. aeruginosa* and *B. cenocepacia* cultures.

Besides β-lactams, we also studied the effect of the fluoroquinolone ciprofloxacin on vesicle secretion in *S. maltophilia*. Ciprofloxacin is believed to induce vesiculation through the SOS response and increased oxidative stress. Increased vesicle secretion was demonstrated in *S. maltophilia* after ciprofloxacin stimulation, but both vesicle morphology and protein cargo showed significant differences to those secreted after β-lactam stress. Two morphologically different populations of ciprofloxacin-induced vesicles were observed: small vesicles (± 50 nm) comparable to β-lactam-induced vesicles and large vesicles (± 100-200 nm) with surface-attached filaments. The protein cargo of ciprofloxacin-induced vesicles includes different stress-related proteins typically induced by DNA damage, protein misfolding and oxidative stress, as well as the ciprofloxacin target DNA gyrase. Remarkably, far more cytoplasmic and inner membrane proteins were identified, indicating the presence of outer-inner membrane vesicles (OIMVs). The presence of both inner and outer membrane was confirmed in the large vesicles with cryo-electron

microscopy. Another interesting observation, was the identification of a cluster of phage tail-related proteins, solely present in ciprofloxacin-induced vesicles. The production of bactericidal phage tails (or tailocins) was already demonstrated in *S. maltophilia* P28, described as maltocin P28. The filaments observed at the vesicle surface are most likely such phage tails. The bactericidal effect of the ciprofloxacin-induced vesicles ratifies this premise. However, among the used strains the bactericidal effect was only observed against *P. aeruginosa* PAO1, not against *S. maltophilia* 44/98 itself or the *B. cenocepacia* type strain.

Dutch summary

Stenotrophomonas maltophilia is een multi-drug resistente nosocomiale pathogeen, die steeds vaker geassocieerd wordt met chronische luchtweginfecties en infecties opgelopen na contact met medische toestellen. De behandeling van *S. maltophilia* infecties wordt bemoeilijkt door zijn diversiteit aan resistentiemechanismen tegen de verschillende antibiotica families zoals β-lactams, aminoglycosiden, quinolones, tetracyclines, macroliden en sulfonamiden.

Voorgaande proteoomstudies aangaande de cellulaire respons van de klinische *S. maltophilia* stam 44/98 toonden een verhoging aan van de expressie van twee buitenste membraan Ax21 homologen na behandeling met het β-lactam imipenem. Ax21 werd eerder al gelinkt aan virulentie en biofilmvorming, en wordt gesecreteerd via buitenste membraan vesikels (*outer membrane vesicles*; OMVs). Wij hebben de cargo van imipenem-geïnduceerde OMVs gekarakteriseerd om inzicht te krijgen in hun mogelijke rol na te gaan in de resistentie tegen β-lactam antibiotica. Proteoomanalyse van de OMVs onthulde de aanwezigheid van een waaier aan eiwitten, voornamelijk buitenste membraan- en periplasmatische eiwitten, maar ook binnenste membraan- en cytoplasmatische eiwitten. Interessant is de identificatie van L1-metallo-β-lactamase en L2-serine-β-lactamase als onderdeel van het OMV proteoom. Wij hebben eveneens het effect nagegaan van *diffusible signal factor* (DSF) quorum sensors op de OMV productie. Een DSF synthase knock-out stam van de nauw verwante plantenpathogeen *Xylella fastidiosa* produceert minder OMVs, wijzend op een inhiberende rol van het DSF systeem. Wij namen verrassend genoeg een toename waar van OMVs in *S. maltophilia* na blootstelling aan zijn endogene DSF, cis-Δ2-11-methyl-dodecaanzuur. Bovendien werd ook een toename in OMV productie geïnduceed na blootstelling aan het *Burkholderia cenocepacia* DSF cis-Δ2-dodecaanzuur. Imipenem, cis-Δ2-11-methyl-dodecaanzuur en cis-Δ2-dodecaanzuur leiden allen tot een abundante OMV-geassocieerde secretie van de twee Ax21 homologen, maar de expressie van beiden lijkt op een verschillende manier gereguleerd. Helaas is de werkelijke rol van Ax21 nog onbekend en dit omvat dus een interessant onderwerp voor toekomstig onderzoek.

Beide chromosomaal gecodeerde β-lactamasen werden geïdentificeerd als onderdeel van de OMV cargo. Daarom werd de intra- en interspecies impact op β-lactam tolerantie van OMVs onderzocht. Met behulp van een nitrocefin β-lactamase assay op intacte OMVs werd een gelijke L1-L2 activiteitsverdeling waargenomen, terwijl de cellulaire activiteit gedomineerd wordt door L1. Daarnaast worden ook de klinisch relevante β-lactams imipenem, amoxicilline en ticarcilline afgebroken door de β-lactamase-bevattende OMVs, weliswaar met verschillende efficiëntie. *S. maltophilia* leeft vaak in polymicrobiële biofilms met species zoals *Pseudomonas aeruginosa* en *Burkholderia cenocepacia*, ondermeer in mucoviscidose-geassocieerde chronische luchtweginfecties. Daarom onderzochten we de mogelijkheid of *S. maltophilia* β-lactamase-bevattende OMVs intra- en interspecies bescherming verlenen tegen β-lactams. De OMVs verhoogden inderdaad niet enkel de β-lactam tolerantie van *S. maltophilia* culturen, maar vertoonden ook een drastisch effect op de tolerantie van de minder resistente *P. aeruginosa* en *B. cenocepacia* culturen. Deze resultaten wijzen op een mogelijks beschermende rol van *S. maltophilia* OMVs tegen β-lactam antibiotica, zowel op intra- als interspecies vlak. In verschillende species werd ook aangetoond dat OMVs belangrijk zijn voor biofilmvorming. Hier werd aangetoond dat β-lactam geïnduceerde vesikels van *S. maltophilia* de vorming van biofilm inhiberen, waarschijnlijk door vrije oppervlakten te blokkeren. Opmerkelijk is dat biofilmvorming gestimuleerd werd na toedienen van deze OMVs aan *P. aeruginosa* en *B. cenocepacia* culturen.

Naast β-lactams werd ook het effect van het fluoroquinolone ciprofloxacine op OMV secretie in *S. maltophilia* bestudeerd. Van ciprofloxacin wordt verondersteld dat het de secretie van vesikels induceert via de SOS respons en verhoogde oxidatieve stress in de cel. Een verhoging in vesikelsecretie werd aangetoond na blootstelling van *S. maltophilia* aan ciprofloxacine, maar de vesikelmorfologie en eiwitcargo verschilt beduidend van de vesikels geïnduceerd na β-lactam stress. Twee morfologisch verschillende populaties van ciprofloxacine-geïnduceerde vesikels werden waargenomen: kleine vesikels (± 50 nm) vergelijkbaar met β-lactam-geïnduceerde vesikels en grote vesikels (± 100-200 nm) met filamenten aan het oppervlak gehecht. De eiwitcargo van ciprofloxacine-geïnduceerde vesikels omvat verschillende stress-gerelateerde eiwitten die doorgaans geïnduceerd worden na DNA schade, verkeerd gevouwen eiwitten,

eiwitten betrokken bij oxidatieve stress, en ook de ciprofloxacine target DNA gyrase. Daarnaast werden opmerkelijk meer cytoplasmatische en binnenste membraan eiwitten geïdentificeerd, wat wijst op de aanwezigheid van buitenste-binnenste membraan vesikels (*outer-inner membrane vesicles*; OIMVs). De aanwezigheid van zowel een binnenste als buitenste membraan werd bevestigd in de grote vesikels met behulp van cryo-elektronen microscopie. Een andere interessante observatie was de identificatie van een cluster van *phage tail* gerelateerde eiwitten, die uitsluitend in de ciprofloxacine-geïnduceerde vesikels voorkomen. De productie van bactericidale *phage tails* (of *tailocins*) werd reeds aangetoond in *S. maltophilia* P28, beschreven als maltocine P28. De filamenten aanwezig op de vesikels zijn hoogstwaarschijnlijk dergelijke *phage tails.* Het bactericidale effect van de ciprofloxacine-geïnduceerde vesikels ondersteunt deze veronderstelling. Onder de gebruikte stammen werd het bactericidaal effect enkel waargenomen tegen *P. aeruginosa* PAO1, niet tegen *S. maltophilia* 44/98 of de *B. cenocepacia* type stam.

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List of abbreviations

2D	two-dimensional
ABC	ATP-binding cassette
ACN	acetonitrile
AMX	amoxicillin
ATP	adenosine triphosphate
AUC	area under curve
BDSF	*Burkholderia cenocepacia* diffusible signal factor
BSA	bovine serum albumin
c-di-GMP	cyclic diguanosine monophosphate
CE	collision energy
CF	cystic fibrosis
CIP	ciprofloxacin
CVA	clavulanic acid
DHFR	dihydrofolate reductase
DHPS	dihydropteroate synthase
DNA	deoxyribonucleic acid
DP	declustering potential
DSF	diffusible signal factor; *Stenotrophomonas maltophilia* DSF
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
FEV	forced expiratory volume
fSPT	fluorescent single particle tracking
GO	gene ontology
ICU	intensive care unit
IM	inner membrane
IPM	imipenem
ISCR	insertional sequence common region

LB	Luria Bertani
LPS	lipopolysaccharide
LTTR	lysR-type transcriptional regulator
MDR	multi-drug resistant
MRM	multiple reaction monitoring
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	mass spectrometry
MWCO	molecular weight cut-off
OD	optical density
OIMV	outer-inner membrane vesicle
OM	outer membrane
OMP	outer membrane protein
OMV	outer membrane vesicle
PABA	p-aminobenzoic acid
PBP	penicillin binding protein
PBS	phosphate buffered saline
PDSF	*Pseudomonas aeruginosa* diffusible signal factor
PEN	penicillin G
PES	polyethersulfone
PG	peptidoglycan
QS	quorum sensing
RNA	ribonucleic acid
RNAP	RNA polymerase
RND	Resistance-Nodulation-Division
rpf	regulation of pathogenicity factor
RPLC	reversed phase liquid chromatography
SMX	sulfamethoxazole
SPT	single particle tracking
TCS	two-component system

TEM	transmission electron microscopy
TET	tetracycline
TIC	ticarcillin
TMP	trimethoprim
TOB	tobramycin
TOF	time-of-flight
UPLC	ultra performance liquid chromatography

Foreword

On the 29th of July 2014, my son Max was born. His birth was anything but easy, especially for the mother. After a very long period of agonizing labour, an alarming drop in the baby's heart rate eventually led to an emergency caesarean. But because of inhaled myconium breathing was difficult, so Max finally ended up in the neonatal intensive care unit (ICU). Luckily, everything turned out fine and today he is a healthy, active toddler.

In his first months on earth, Max received several courses of antibiotics. During his time in the neonatal ICU he got a few preventive, intravenous antibiotic treatments because of the risk of lung infection. After a few month he also had a reoccurring infection in his belly button, treated cutaneously with mupirocine, bacitracin and polymyxin B. And later he suffered from a persistent ear infection, which was first treated with amoxicillin, and later with amoxicillin-clavulanic acid due to antibiotic resistance. So during his half year of life, he was exposed to several antibiotic treatments. I do not know how things would have turned out without the antibiotics, and the same question can be asked for all of our lives. On the other hand, one could wonder what impact it had (or still has) on his microbiome. We are living in a bacterial world, and bacteria are inextricably connected to our bodies. Although antibiotics improved the quality of human life and drastically prolonged the average human life span, its use is not without danger.

Introducing antibiotics in our bodies may kill harmfull, pathogenic bacterial species, but also harmless or beneficial bacterial species. Especially when broad-spectrum antibiotics are used. The notable increase in several modern diseases like obesity, asthma, and juvernile diabetis are believed to be linked to the overuse of antibiotics (research regarding this topic is nicely described in the book 'Missing microbes: How the overuse of antibiotics is fueling our modern plagues' by Martin J. Blaser). Antibiotic overuse and misuse also enhances the selection of antibiotic resistant species due to selective pressure. And the resulting microbiome dysbiosis could create opportunies for other harmfull bacteria to take their place, above all when these species are resistant to the antibiotic compound that is used. The rise of multi-drug resistant (MDR) species

is truly problematic for public health and is compromising our optimistic view on treating bacterial infections.

The scientific world agrees that antibiotics should be used in a more sensible manner to prevent the selection of MDR species, and that the knowledge about resistance should be broadened to develop new, more efficient ways of treating (MDR) infections. This doctoral dissertation reports on specific resistance mechanisms of the emerging MDR pathogen *Stenotrophomonas maltophilia*, one of many difficult-to-treat species due to its many intrinsic and acquired resistance strategies.

Introduction

- Chapter 1 -

The multi-drug resistant nosocomial species *Stenotrophomonas maltophilia*

1.1 *Stenotrophomonas maltophilia* is an emerging multi-drug resistant pathogen

Stenotrophomonas maltophilia is a non-fermenting Gram-negative environmental bacterium, of which some multi-drug resistant (MDR) strains are increasingly isolated from clinical settings as nosocomial pathogens (Brooke, 2012). *S. maltophilia* was initially classified as a member of the *Pseudomonas* genus, then of the *Xanthomonas* genus, until it was finally classified as a member of the *Stenotrophomonas* genus (Abbott et al., 2011) (Figure 1).

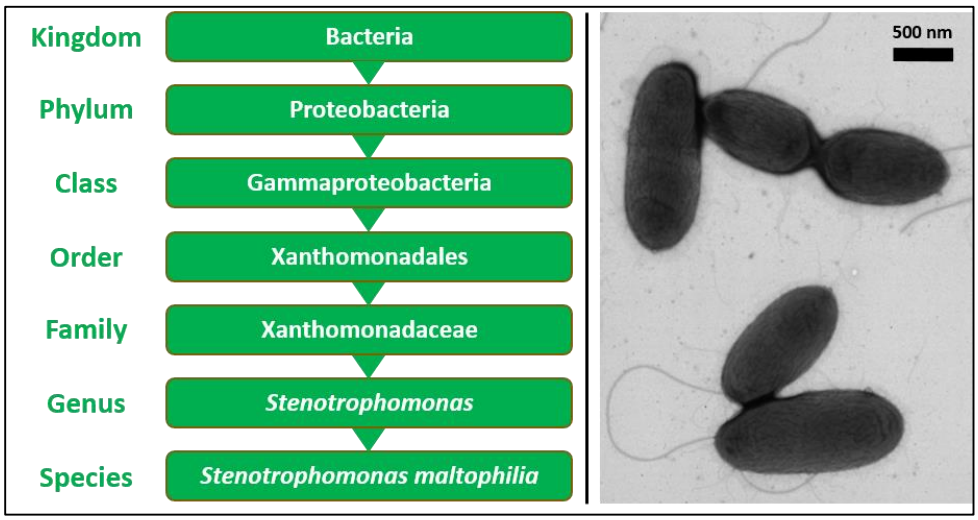


Figure 1. Taxonomic classification of *Stenotrophomonas maltophilia* (**left**) and microscopy image of *S. maltophilia* cells (**right**) (picture: Wouter Van Putte, Riet De Rycke).

S. maltophilia is growingly associated with human respiratory tract infections. In Europe, it now belongs to the ten most frequently isolated microorganisms in intensive care unit (ICU) acquired pneumonia (European Centre for Disease Prevention and Control, 2011). In Belgium, approximately 5-6 % of ICU acquired pneumonia cases are associated with this organism, ranking number five after *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli* and *Enterobacter cloacae* (WIV, 2012). *S. maltophilia* is also one of the emerging MDR organisms in the lungs of cystic fibrosis (CF) patients (Amin and Waters, 2014; Parkins and Floto, 2015). Furthermore, it is involved in bacteraemia, skin and soft tissue infections and urinary tract infections (Brooke, 2012). These infections are characterized by a high mortality rate due to its intrinsic and acquired resistance to many of the current antibiotics, and the ability to form biofilm

(Sánchez, 2015). The genome of the pathogenic *S. maltophilia* k279a strain is sequenced, and indicates an organism that is well adapted for living in antimicrobial environments (Crossman et al., 2008). It contains resistance genes to almost all classes of β-lactams, such as penicillins, cephalosporins and carbapenems, but also against a variety of other antibiotics like aminoglycosides, macrolides, quinolones, tetracyclines and sulfonamides (Brooke, 2012).

1.2 Virulence, colonization and infection

In most cases, *S. maltophilia* is associated with persistent airway colonization and chronic infection, among which also in patients with CF. Although it is still uncertain whether *S. maltophilia* plays a true pathogenic role in these patients, its presence could be linked to a profound state of lung disease and can influence clinical outcome (Hansen, 2012).

The pathogenesis of this organism is not well understood. Few clinical strains produce true toxins (Adamek et al, 2014). However, all clinical strains sequenced so far contain a number of putative virulence factors. They produce flagella, fimbriae, pili, fimbrial adhesins, and lipopolysaccharides (LPS), all having immunostimulatory effects that may induce inflammation (Abbott et al., 2011). Moreover, these virulence factors are involved in biofilm formation, increasing the protection against antibiotics and host immunity and enhancing spread along surfaces (Pompilio et al., 2010). Other potential virulence genes encode for adhesins, hemolysin, proteases, phospholipases and hemoglobin binding proteins that may contribute to cell or tissue damage (Adamek et al., 2014). Another important factor in *S. maltophilia* virulence is diffusible signal factor (DSF) mediated cell-cell communication, involved in regulating different virulence responses and antibiotic resistance (Ryan and Dow, 2010).

The attachment and biofilm growth of *S. maltophilia* on abiotic surfaces is a serious problem in hospital settings. Colonized medical devices often lead to severe device-associated nosocomial infections like bacteraemia, urinary tract infection and pocket infections (Passerini de Rossi et al., 2007). Healthcare associated sources of *S. maltophilia* include intravenous and urinary catheters,

nebulizers, dialysis machines, thermometers, ventilator circuits, and medical implants (Abbott et al., 2011) (Figure 2).

Figure 2. Pacemaker pocket infection caused by *Stenotrophomonas maltophilia* (Aktuerk et al., 2014).

1.3 Treatment and resistance

The multi-drug resistance poses a threat for treating *S. maltophilia* infections, narrowing down the number of effective antibiotic compounds available. Also, being an intrinsically resistant environmental species, it can provide other pathogenic species with resistance genes via horizontal gene transfer (Sánchez, 2015).

Most clinical isolates are not susceptible to β-lactam antibiotics like imipenem, meropenem or amoxicillin, even in combination with the β-lactamase inhibitor clavulanic acid (e.g. Amoxiclav, Augmentin). Ticarcillin-clavulanic acid is the most effective β-lactam antibiotic treatment against *S. maltophilia*, but resistance is on the rise (Abbott et al., 2011). The preferred treatment of *S. maltophilia* involves the bacteriostatic drug sulfamethoxazole-trimethoprim (SMX-TMP), or alternatively newer fluoroquinolones (e.g. moxifloxacin) and tetracycline derivatives (e.g.

tigecycline) (Brooke, 2012). However, resistance against these compounds is emerging, so new treatments typically include different antibiotic combinations that work in synergy. For example, SMX-TMP was shown to be more effective against *S. maltophilia* when used in combination with either ciprofloxacin, ceftazidime or tobramycin, than SMX-TMP administered alone (Zelenitsky et al., 2005).

Interestingly, when *S. maltophilia* isolates from CF and non-CF patients were compared, the CF strains showed significantly higher resistance towards different antibiotics (e.g. SMX-TMP, ciprofloxacin) (Cantón et al., 2003). Most alarming, a study on CF sputum samples showed that only about one third of isolates were susceptible to SMX-TMP, only one fourth to ciprofloxacin, and all were resistant to imipenem (Valenza et al., 2008).

1.4 Antibiotic resistance mechanisms

After exposure to β-lactam compounds, *S. maltophilia* immediately starts expressing two chromosomal genes encoding β-lactamase enzymes. L1-β-lactamase is a Zn^{2+}-dependent (Ambler class B) metallo-β-lactamase, and L2-β-lactamase is a (Ambler class A) serine-β-lactamase (Senol, 2004) (Figure 3). The gene encoding for L2 (bla_{L2}) is located in an operon together with the gene for the LysR-type transcriptional regulator (LTTR) AmpR (Yang et al., 2009), comparable with the AmpR-AmpC module which is widespread in various *Enterobacteriaceae* like *Citrobacter fruendii* and *Escherichia coli* (Jacobs et al., 1997), and also in *Pseudomonas aeruginosa* (Moya et al., 2009). In this AmpR-AmpC system the induction of the chromosomally encoded *ampC* gene (coding for a class A serine-β-lactamase) is intimately linked to the peptidoglycan (PG) recycling pathway (Park and Uehara, 2008). The L1 gene (bla_{L1}) is not associated with an LTTR binding motif, despite the fact that AmpR is necessary for its expression (Lin et al., 2009). The L1 β-lactamase has a much broader specificity, and it is not susceptible to clavulanic acid, a β-lactamase inhibitor that is often used in combination with β-lactam therapeutics to counteract the induced resistance response (Mercuri et al., 2002).

Another important mechanism contributing to multi-drug resistance in *S. maltophilia* is the production of different multidrug efflux pumps (Brooke, 2012) (Figure 3). Efflux pumps are protein complexes capable of extruding a broad range of chemical compounds from the cell (Sun et al., 2014). *S. maltophilia* strains have different gene clusters encoding Resistance-Nodulation-Division (RND) efflux pumps. These transporters are typically tripartite complexes spanning the inner and outer membrane of the cell wall, and actively pump out toxic compounds (Delmar et al., 2014). For example, the SmeABC and SmeDEF efflux pumps play an important role in antibiotic resistance in *S. maltophilia* by eliminating antibiotics like tetracyclins, (fluoro)quinolones, aminoglycosides, and even β-lactams (Li et al., 2002; Chang et al., 2004). While the RND pumps are driven by the proton motive force, multidrug ATP-binding cassette (ABC) transporters use ATP for compound translocation. *S. maltophilia* produces the multidrug ABC transporter SmrA, which establishes resistance to ciprofloxacin, norfloxacin and tetracycline derivates (Al-Hamad et al., 2009).

Resistance against some antibiotics can also be established by direct enzymatic modification, hereby inactivating the antibiotic compound (Figure 3). For example, aminoglycosides can be modified by the acetyltransferase AAC(6')-Iz or by the phosphotransferase Aph(3')-IIc, increasing resistance against compounds like kanamycin, neomycin and tobramycin (Li et al., 2003; Okazaki and Avison, 2007). Besides modifying the antibiotic compound itself, the target site can also be modified to avoid interaction and inhibition. This strategy is used in the resistance against fluoroquinolone type antibiotics, with the modification of DNA gyrase and topoisomerases (Sánchez and Martinez, 2010).

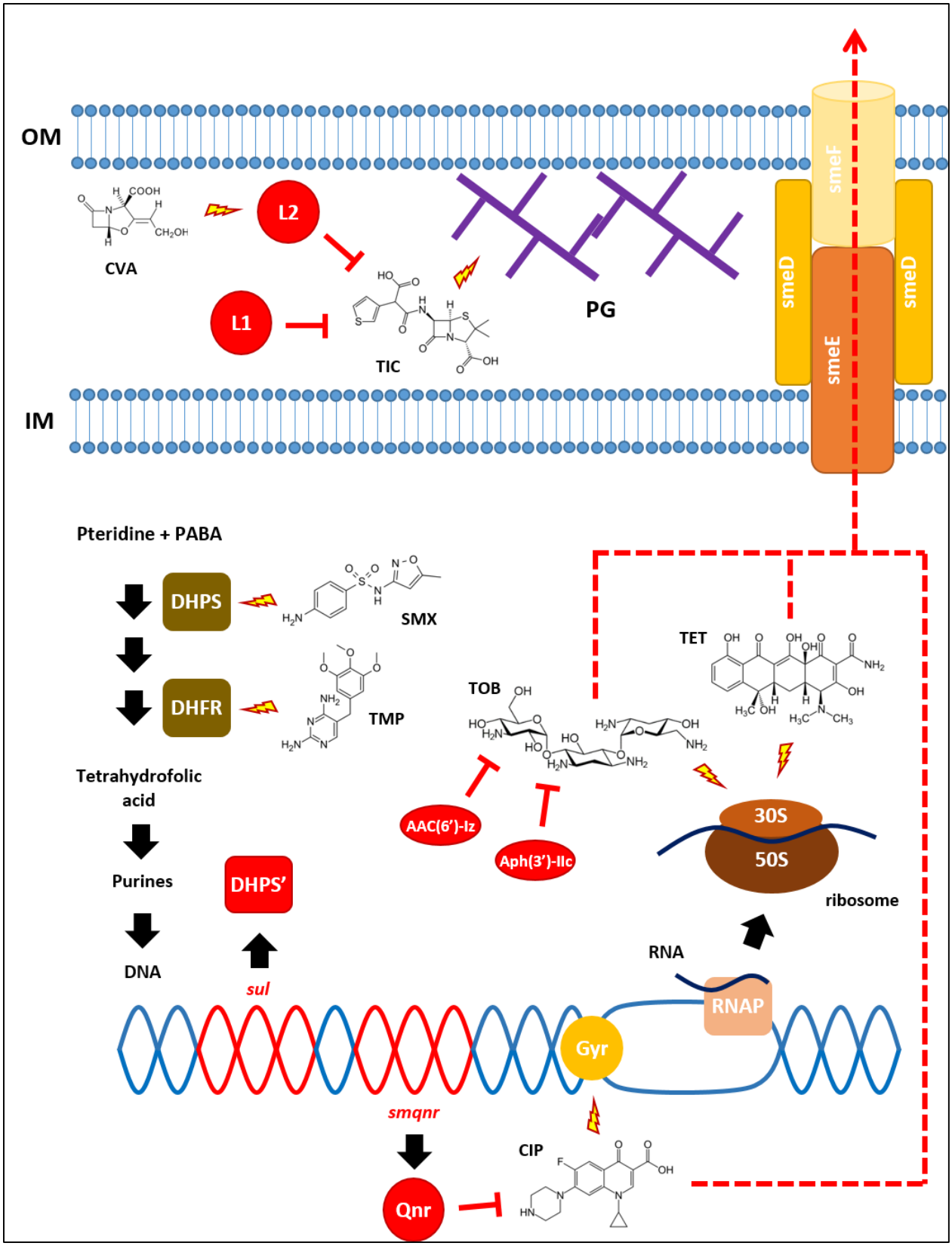
A potent drug recommended for treating *S. maltophilia* infections is the folic acid biosynthesis inhibitory drug SMX-TMP. However, resistance against SMX-TMP is emerging because of the acquisition of alternative dihydropteroate synthase (DHPS) genes, or *sul* genes (Byrne-Bailey et al., 2009) (Figure 3). The *sul* resistance genes can be acquired through class 1 integrons (*sul1*), or through transposon-like structures located on plasmids (sul2) with the possibility of inter- and intra-generic transfer through insertional sequence common region (ISCR) elements (Hu et al.,

2011; Chung et al., 2015). Instead of producing an alternative protein that is not recognized by the antibiotic compound, the target site can also be shielded from antibiotic interaction. *S. maltophilia* possesses Sm*qnr* genes that encode for Qnr pentapeptide repeat proteins that protect DNA gyrase and topoisomerases from quinolone inhibition (Gordon and Wareham, 2010) (Figure 3).

1.5 Biofilm formation in *S. maltophilia*

The susceptibility of *S. maltophilia* to antibiotics is also largely affected by its capacity to grow as a biofilm. Biofilms are microorganism communities, consisting of single or multiple species that are growing attached to a surface (biotic or abiotic) (O'Toole et al., 2000). The initiation of biofilm formation is characterized by the attachment of cells to a surface, a process that is highly dependent on flagella (motility) and fimbriae (attachment) (Van Houdt and Michiels, 2005). The fimbriae-1 protein (SMF-1) plays a key role in the attachment of *S. maltophilia* cells, and subsequent biofilm formation (de Oliveira-Garcia et al., 2003).

The biofilm mode of growth can aid in antibiotic resistance in different ways. First of all, the antibiotic compounds show limited penetration of the biofilm, making treatment far less efficient (Trautner and Darouiche, 2004). Compounds that do reach the cells inside the biofilm are immediately confronted with a high density of cells potentially equipped with different resistance mechanisms (Jolivet-Gougeon and Bonnaure-Mallet, 2014). The antibiotic stress also leads to an increase in mutagenesis and horizontal gene transfer, stimulating the acquisition and spread of resistance genes inside the biofilm (Wenzel, 2007). However, studies regarding the behavior of *S. maltophilia* inside biofilms, especially concerning antibiotic resistance and horizontal gene transfer, are scarce.


OM
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CVA
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TMP
Tetrahydrofolic acid
Purines
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DHPS'
sul
TOB
TET
AAC(6')-Iz
Aph(3')-IIc
30S
50S
ribosome
RNA
RNAP
Gyr
smqnr
Qnr
CIP

Figure 3. Overview of important resistance mechanisms of *Stenotrophomonas maltophilia*. Ticarcillin (TIC) is hydrolyzed by the L1- and L2-β-lactamases, of which only L2 is susceptible to the β-lactamase inhibitor clavulanic acid (CVA). The DNA gyrase (Gyr) inhibitor ciprofloxacin (CIP) can become ineffective by blocking the gyrase CIP binding site with the Qnr pentapeptide (encoded by the *smqnr* gene), or by removing CIP via the SmeDEF efflux pumps. Tetracycline (TET) and tobramycin (TOB) are removed via the SmeDEF efflux pump, TOB can be modified by acetyltransferase AAC(6')-Iz or phosphotransferase Aph(3')-IIc. Trimethoprim (TMP)-sulfamethoxazole (SMX) inhibition of dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) respectively, can be overcome by producing alternative DHPS' proteins (encoded by *sul* genes). OM: outer membrane; PG: peptidoglycan; IM: inner membrane; RNAP: RNA polymerase; PABA: p-aminobenzoic acid.

1.6 Cell-cell communication and quorum sensing in *S. maltophilia*

It is now well established that bacterial cell-cell communication is of great importance, if not essential, for species survival. Bacteria release all kinds of small molecules into the environment, such as antibiotics, siderophores and metabolites that can function as cell-to-cell signaling molecules (Williams, 2007). A particular set of these cell-cell communication molecules are involved in a process called 'quorum sensing' (QS), which allows bacteria to monitor the population density and behave accordingly.

In general, Gram-negative bacteria use acylated homoserine lactones. However, some species use another QS system that involves fatty acid diffusible signal factors (DSF). DSF was first described in the plant pathogen *Xanthomonas campestris* as a *cis*-unsaturated fatty acid that activates a cluster of 'regulation of pathogenicity factor' (*rpf*) genes (Barber et al., 1997; Ryan and Dow, 2011). The *rpf* cluster is conserved throughout the Xanthomonads, including *S. maltophilia* (Fouhy et al., 2007). Structurally similar DSF molecules are also produced by other important pathogens like *Burkholderia cenocepacia* (Boon et al., 2008) and *Pseudomonas aeruginosa* (Davies and Marques, 2009). DSF molecules produced by those human pathogens are involved in regulating virulence, biofilm formation, and antibiotic resistance, and some signals can even influence the behavior of non-producing species (Ryan et al., 2015) (Table 1). In particular, the *S.*

maltophilia DSF cis-Δ2-11-methyl-dodecenoic acid has a broad specificity, influencing the behavior of both *P. aeruginosa* and *B. cenocepacia*, e.g. by inducing biofilm formation (Ryan et al., 2008). This cross-species effect of DSF has physiological implications, since these three species are often found together, for example in the lungs of patients with cystic fibrosis (CF) (Twomey et al., 2012).

Table 1. Factors regulated by DSF family signal in different pathogens (adapted from Ryan et al., 2015).

Organism	Biological response
Xanthomonas campestris	Extracellular enzyme production; extracellular polysaccharide production; PilA-dependent motility; biofilm dispersal; small RNA synthesis; elongation factor P turnover; cyclic glucan synthesis; *Arabidopsis* stomatal opening factor(s)
Xanthomonas oryzae pvs. *oryzae and oryzicola*	Iron uptake; iron-dependent virulence to rice; extracellular proteases; extracellular polygalacturonase, asparagine synthase
Xanthomonas axonopodis	Extracellular enzyme production; extracellular polysaccharide production; flagellar-dependent biofilm formation
Xanthomonas citri subsp. *citri*	Host leaf surface adherence and penetration in lemon and grapefruit leaves
Xanthomonas hortorum pv. *pelargonii*	Virulence; in planta movement
Xylella fastidiosa	Insect vector colonization and attachment; transmission to plants; outer membrane vesicle release; extracellular enzyme production
Pseudomonas aeruginosa	Biofilm dispersal; reversal of dormant persister cell state
Burkholderia cenocepacia	Adherence, biofilm formation and swarming motility; virulence in mouse, zebrafish and insect models
Stenotrophomonas maltophilia	Heavy metal tolerance and antibiotic resistance; extracellular protease production; virulence to *Galleria mellonella* larvae

In *S. maltophilia*, as in all DSF producing Xanthomonads, DSF signaling is regulated by the Rpf proteins (regulation of pathogenicity factors) (Figure 4). DSF synthesis, mainly cis-Δ2-11-methyl-dodecenoic acid, is achieved by the RpfF DSF synthase (enoyl-CoA hydratase family enzyme) and RpfB (long-chain fatty acyl coenzyme A ligase) (Fouhy et al., 2007; Cheng et al, 2010; Deng et al,

2011). Cellular perception and signaling occurs through the two-component system (TCS) histidine sensor kinase RpfC. Upon binding with DSF at its periplasmic domain, RpfC gets autophosphorylated on a conserved histidine residue. This phosphate is passed onto an aspartic acid residue of the RpfG TCS response regulator. Consequently, the activated RpfG, containing a HD domain, is then acting as a cyclic diguanosine monophosphate (c-di-GMP) phosphodiesterase, catalyzing c-di-GMP degradation (Ryan and Dow, 2011). The c-di-GMP secondary messenger is important in linking environmental signals and subsequent alterations in cellular functions, like biofilm formation and virulence (Ryan, 2013). Moreover, in the absence of DSF, the RpfF synthase is interacting with RpfC and remains inactive. When DSF binds to the RpfC receptor, auto-stimulation of its biosynthesis is initiated because RpfF is released and activated (Cheng et al., 2010). The genes of these proteins are all located in the *rpfBFCG* gene cluster. A recent study of Huedo et al. (2014) analyzed the *rpf* gene cluster in 82 *S. maltophilia* clinical isolates, and demonstrated the presence of two different *rpf* cluster populations. The two populations show variation in the N-terminal regions of proteins RpfF and RpfC. One of the populations exhibited a DSF-deficient phenotype, due to a permanent repression of RpfF by RpfC.

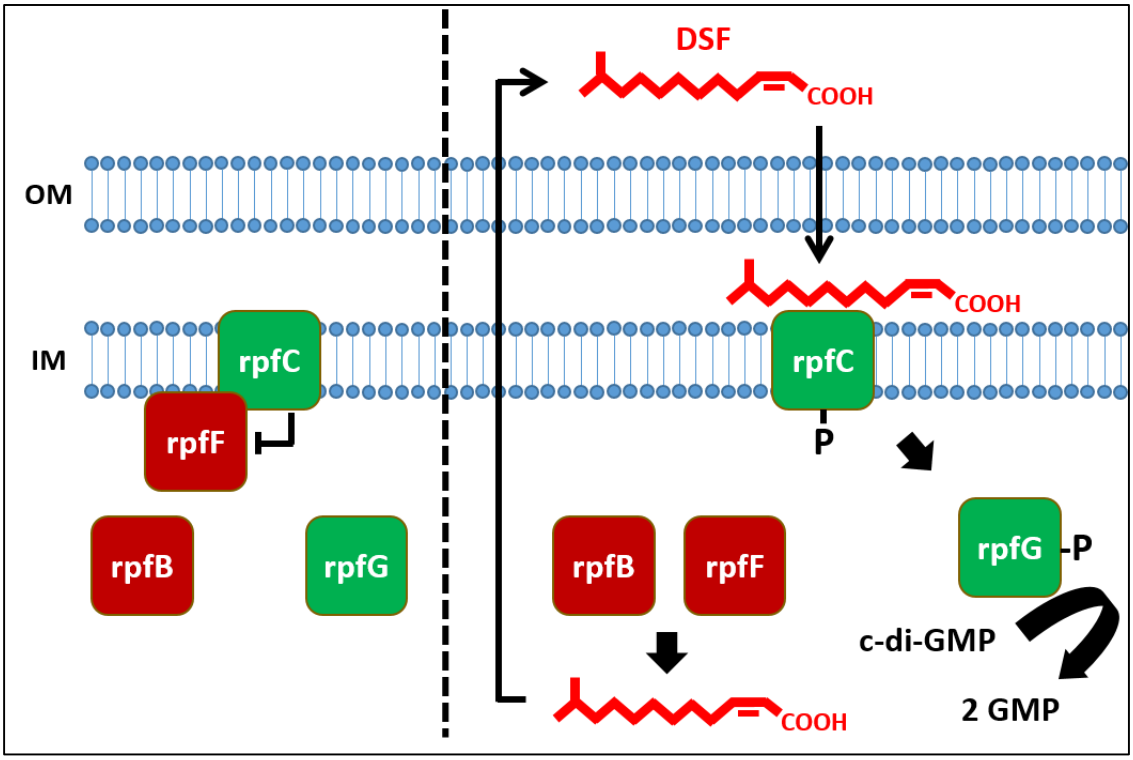

Figure 4. Diffusible signal factor signalization. In the absence of cis-Δ2-11-methyl-dodecenoic acid (DSF), the DSF synthase rpfF is inhibited by the two-component system (TCS) histidine kinase receptor rpfC (**Left**). When DSF binds to rpfC, rpfF is released and synthesizes DSF. The activated rpfC gets autophosphorylated and passes the phosphoryl group to the TCS response regulator rpfG. The activated rpfG catalyzes c-di-GMP degradation (**Right**).

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- Chapter 2 -

Outer membrane vesicle mediated resistance in Gram-negative bacteria

2.1 The Gram-negative bacterial cell wall

The cell wall of Gram-negative bacteria is composed of an inner, cytoplasmic membrane and an outer membrane, with a periplasmatic peptidoglycan (PG) layer in between (Figure 5A; Schwechheimer and Kuehn, 2015). While the inner membrane is composed of a bilayer of phospholipids, the outer membrane consists of an inner phospholipid layer and an outer lipopolysaccharide (LPS) layer. Peptidoglycan is a N-acetylglucosamine-N-acetylmuramic acid (NAG-NAM) disaccharide polymer, most often interconnected with tetrapeptide side chains (such as L-alanine-D-glutamic acid-meso-diaminopimelic acid-D-alanine). The cell wall is further stabilized via different PG-protein interactions: examples are the covalent interaction with the Lpp lipoprotein, and non-covalent interactions with outer membrane protein A (OmpA) and the Tol-Pal protein complex spanning the inner membrane, the PG layer and the outer membrane.

The PG layer is a crucial part of the cell wall, as it maintains cell shape and strength (Typas et al., 2012). Its biosynthesis is initiated by the production of the UDP-bound NAM-pentapeptide, produced in the cytoplasm by a series of Mur proteins, which is eventually transferred to an undecaprenol lipid carrier in the cytoplasmic membrane (Lipid I). Then NAG is added (Lipid II) and the NAG-NAM-pentapeptide is flipped to the periplasmic side of the membrane. There it is connected to the growing glycan chain by a glycosyl transferase (GTase) and the peptide side chain is crosslinked with other peptide side chains or with Lpp by a transpeptidase (TPase). Peptide side chains can be trimmed by carboxypeptidases (CPase) and crosslinks cleaved by endopeptidases (EPase), and the glycan chain can be cleaved by endo- or exo-lytic transglycosylases (LT). A schematic representation of PG synthesis and cleavage is depicted in Figure 5B (Typas et al., 2012).

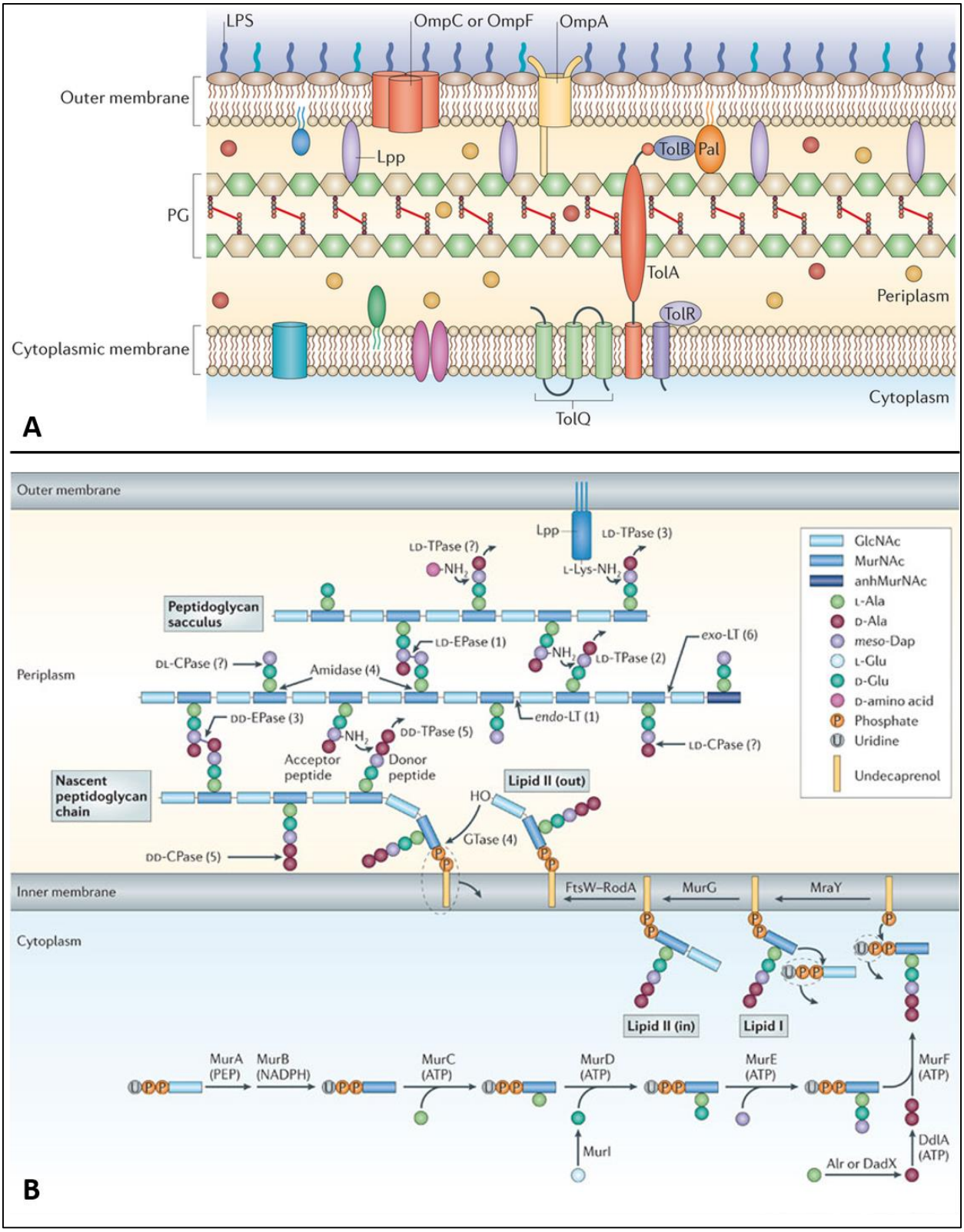

Figure 5. (A) Structure of the Gram-negative cell wall (Schwechheimer and Kuehn, 2015). **(B)** Synthesis and processing of peptidoglycan (Typas et al., 2012)

2.2 Outer membrane vesicles

Outer membrane vesicles (OMVs) are secreted spheroid membrane nanoparticles (20-300 nm), formed through outer membrane budding. They encapsulate cell material and pinch off into the extracellular environment (Mashburn-Warren et al., 2008; Bonnington and Kuehn, 2014) (Figure 6). OMV secretion is conserved in Gram-negative bacteria, both pathogenic and non-pathogenic (Kuehn and Kesty, 2005). OMVs are involved in important biological processes including virulence, pathogenesis, cell-cell communication, biofilm formation and antibiotic resistance (Olsen and Amano, 2015). The secretion of proteins, DNA/RNA, or other compounds as OMV cargo offers various advantages as opposed to 'naked' secretion, i.e. protection against degradative enzymes, enabling long-distance transport, efficient delivery by adhering to and interacting with target host cells, and the instant delivery of concentrated cargo packages (Bonnington and Kuehn, 2014).

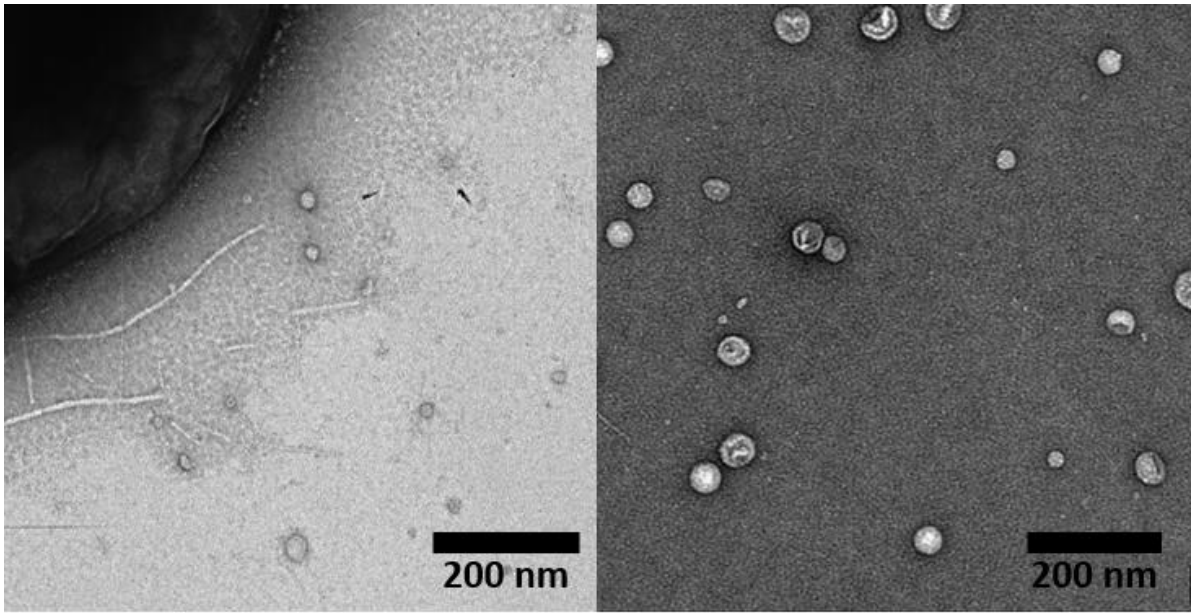

Figure 6. Microscopy image of OMV secretion by *Stenotrophomonas maltophilia* (**left**), and purified *S. maltophilia* OMVs (**right**) (pictures: Wouter Van Putte, Riet De Rycke).

2.3 OMV biogenesis

The biogenesis of OMVs and cargo selection is not yet entirely understood, but it is believed to be steered by an active process (Haurat et al., 2014). A passive formation of OMVs would entail an equal distribution of outer membrane proteins in the OMVs and the cell wall. Since this is not always the case, OMV formation is therefore potentially limited to specific areas on the cell surface, and/or differences exist in protein targeting towards the vesicles. Several studies have demonstrated the unequal OM-OMV distribution of proteins (Lappann et al., 2013; Aguilera et al., 2014; Jang et al., 2014).

The secretion of OMVs would require a local disturbance in PG-OM crosslinking and the induction of membrane curvature (Haurat et al., 2014; Schwechheimer and Kuehn, 2015). The outer membrane lipoprotein Lpp seems to play a crucial role in the OMV biogenesis process. Lpp is an abundant lipoprotein, responsible for a large part of PG-OM crosslinks, and a reduction of Lpp-PG crosslinks leads to higher OMV production (Schwechheimer et al., 2015). However, increased OMV secretion can be induced independent of the number of Lpp-PG crosslinks. The accumulation of envelope components, for example PG degradation products or LPS, can potentially displace crosslinks and cause membrane bulging (Schwechheimer et al., 2014). Furthermore, the lipid composition of the outer membrane also plays a part in OMV biogenesis. Outer membrane lipid domains enriched with charged LPS types and/or unsaturated and branched fatty acids can lead to localized membrane bulging due to charge repulsion or increased membrane fluidity, resulting in increased OMV production (Schwechheimer and Kuehn, 2015; Tashiro et al., 2011; Murphy et al., 2014). Finally, OMV biogenesis is also associated with the formation of fimbriae. Deletion of the fimbrial fimA protein in *Porphyromonas gingivalis* led to a significant reduction in OMV secretion (Mantri et al., 2015). The question remains whether these fimbriae directly cause vesicle production, or if a difference in cell wall integrity between fimbriated and afimbriated cells is causing vesiculation.

Cell stress contributes in part or directly to OMV biogenesis. For example, β-lactam antibiotics inhibit penicillin binding proteins (PBPs), causing a reduction in PG crosslinking and the accumulation of PG degradation products in the periplasm. As mentioned above, these effects directly lead to increased OMV secretion. Other types of cell stress (e.g. oxidative stress, other types of antibiotics) provoke the accumulation of misfolded proteins in the periplasm, which could likewise stimulate vesicle production. Alternatively, extracellular signals can activate signaling cascades that indirectly lead to alterations in PG-OM crosslinking and/or OM LPS composition. For example, a link was established between the DSF QS system and OMV biogenesis in the plant pathogen *Xylella fastidiosa* (Ionescu et al., 2014). The deletion of the *rpfF* DSF synthase gene led to a significant increase in OMV secretion. This study implies a possible regulatory role for the DSF system, either directly or via the impact it has on the downstream pool of the c-di-GMP secondary messenger.

2.4 OMV secretion and antibiotic resistance

OMVs can actively contribute to antibiotic resistance via different modes (Schwechheimer and Kuehn, 2015). OMVs can serve as decoy 'cells' to bind or capture the antibiotic compound, or can transport antibiotics back to the outside of the cell (Figure 7, A). Next, OMVs have been reported to destroy the antibiotic extracellularly by the packing of antibiotic degrading enzymes inside the OMVs (Figure 7, B). Also, OMVs are known to carry resistance genes aiding in the spread of resistance (Figure 7, C).

The presence of β-lactamases in secreted OMVs was demonstrated in different species, for example in *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Moraxella catarrhalis*, group A Streptococci, and *Bacteroides* spp. (Ciofu et al., 2000; Schaar et al., 2011; Lee et al., 2013; Stentz et al., 2015). The OMV-associated β-lactamases were shown to be biologically active and capable to provide protection against β-lactam antibiotics. The mode of action of β-lactam antibiotics, and the periplasmic allocation of β-lactamases, raises the question whether the OMV-associated secretion of the β-lactamase enzymes is merely accidental or predestined. Nevertheless the

extracellular implications remain valid. Not only the enzymes, but also β-lactamase genes can be secreted through OMVs, potentially leading to horizontal gene transfer. For example, carbapenem-resistant *Acinetobacter baumanni* strains are able to secrete the plasmid-borne bla_{OXA-24} gene via OMVs, and protect carbapenem-susceptible *A. baumanni* strains (Rumbo et al., 2011).

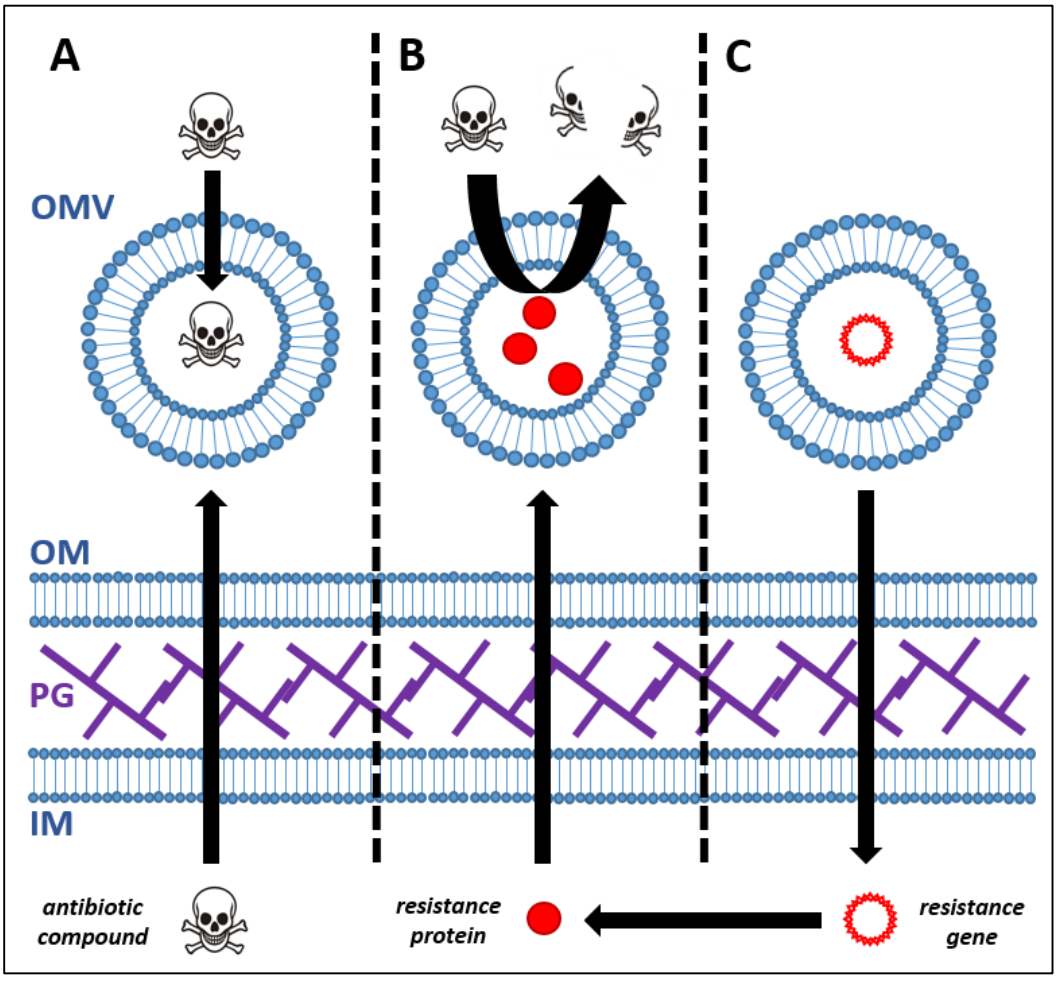

Figure 7. Possible roles of OMVs in antibiotic resistance. OMVs can be used for exporting antibiotic compounds, or to capture antibiotic compounds extracellularly (**A**). OMVs can carry antibiotic degrading enzymes (**B**), and can assist in the transmission of resistance genes (**C**). OMV: outer membrane vesicle, OM: outer membrane, PG: peptidoglycan, IM: inner membrane.

However, other antibiotic types (non-β-lactam) can also increase OMV secretion, meaning that the process is not only achieved through cell wall stress *stricto senso*, but potentially through

alternative regulatory mechanisms (e.g. through the DSF QS system). In *P. aeruginosa* it was shown that the fluoroquinolone ciprofloxacin induced vesiculation as part of the SOS response provoked by this type of antibiotic (Maredia et al., 2012). This phenomenon was also observed in *Acholeplasma laidlawii*, where the vesicles mediate export of nucleotides containing the antibiotic target gene, as well as the ciprofloxacin itself (Medvedeva et al., 2014).

2.5 OMV and biofilm formation

An important feature of many pathogens is the ability to grow as part of a biofilm community. It provides protection against antimicrobial treats, enables coordinated responses to environmental changes, and promotes the spread of antibiotic resistance. OMVs were proven to be important constituents of biofilm. About 20% of the *P. aeruginosa* biofilm extracellular matrix proteome was shown to be common to OMV protein content (Couto et al., 2015). Remarkably, there are indications that biofilm associated OMVs differ considerable from planktonic OMVs (Schooling and Beverdige, 2006). Considering the aforementioned presence of β-lactamases and β-lactamase genes in OMVs, this could potentially increase the antibiotic resistance of biofilms, and the spread of resistance genes within the biofilms, respectively.

OMVs are known to influence cell aggregation, surface attachment, and biofilm formation as well. However, a duality exist on the intra- or inter-species effect that these vesicle have on biofilm formation. In some cases OMVs stimulate biofilm formation, while in others they inhibit biofilm formation. For example, the OMVs from *Porphyromonas gingivalis* negatively influence the formation of *Streptococcus gordonii* biofilm, but stimulate *Staphylococcus aureus* biofilm formation (methicillin-resistant *S. aureus*, MRSA, included) (Ho et al., 2015). The plant pathogen *Xylella fastidiosa* secretes OMVs to block the surfaces in plant xylem vessels leading to deeper plant colonization (Ionescu et al., 2014). In contrast, OMVs from *Helicobacter pylori* stimulate biofilm formation by initiating cell aggregation (Yonezawa et al., 2009). So, most likely depending on the OMV cargo, these vesicles can either inhibit biofilm formation by blocking the available surfaces, or stimulate biofilm formation through OMV-mediated cell aggregation (Figure 8).

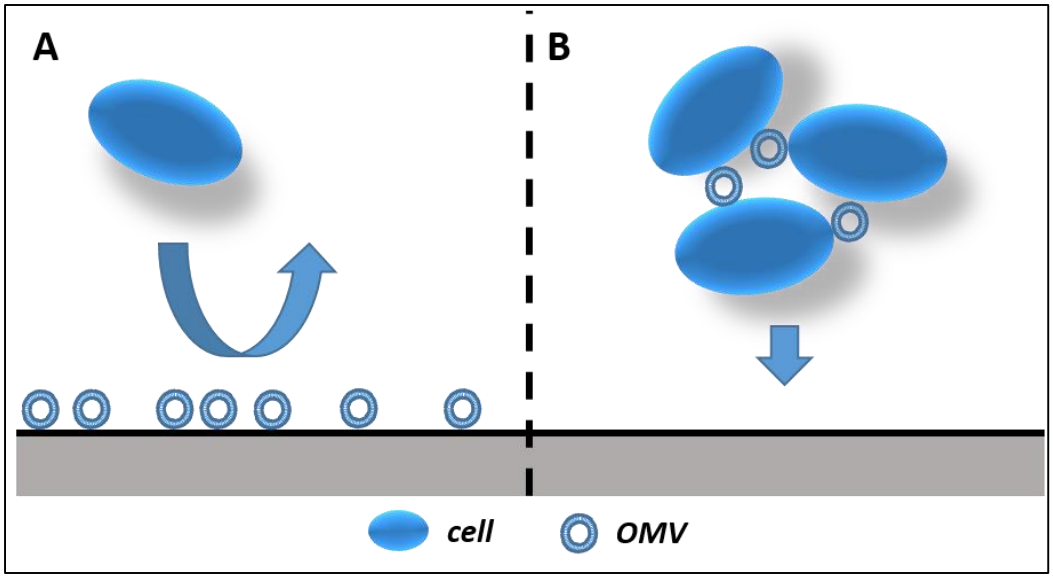

Figure 8. The effects of OMVs on biofilm formation. OMVs blocking surfaces hereby inhibiting cell attachment and biofilm formation (**A**), and OMVs inducing cell aggregation and biofilm formation (**B**).

2.6 OMVs in bacterial communities

OMVs can have different roles in bacterial communities. As mentioned above, by acting as decoy targets for antibiotics or by extracellularly degrading antibiotics, the OMVs can in certain conditions be considered as public goods that benefit the bacterial community (Schwechheimer and Kuehn, 2015). Furthermore, OMVs can facilitate community nutrient acquisition through the packing of proteases and glycosidases into vesicles (Elhenawy et al., 2014). Furthermore, many studies report on the important role of OMVs in iron acquisition in different species. For example, the capturing of iron by OMV associated iron acquisition proteins, and the delivery of iron back to the cells, was demonstrated in *Mycobacterium tuberculosis* (Prados-Rosales et al., 2014).

Next to beneficiary roles of OMVs in bacterial communities, there are reports on harmfull effects as well. For example, *Myxococcus xanthus* uses OMVs to lyse *E. coli* and preys on its liberated cell content (Evans et al., 2012). When *P. aeruginosa* is treated with the aminoglycoside gentamicin, it secretes gentamicin- and peptidoglycan hydrolyse-containing membrane vesicles with bactericidal effects against *B. cenocepacia* group IIIa (Allen and Beveridge, 2003). Another study

reports on the association of membrane vesicle secretion and the production of pyocin (a phage related tailocin bacteriocin) in *P. aeruginosa* as a response to stress (Toyofuku et al., 2014; Ghequire et al., 2015). However, the knowledge concerning the OMV-mediated secretion of bacteriocins for competitive purposes is limited.

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Research aim

Stenotrophomonas maltophilia is well known for its ability to resist different types of antibiotics. It is considered as a natural reservoir of resistance genes: environmental fresh water isolates do not differ significantly from clinical isolates. Its genome is sparked with transposable genetic elements, and therefore *S. maltophilia* is claimed to be at the origin of spreading resistance genes. As it is emerging as an opportunistic nosocomial pathogen, the host laboratory started to use this species as a model organism to study resistance mechanisms. It uses the *S. maltophilia* 44/98 strain that was isolated from a respiratory secretion from an ICU patient at the Varese University Hospital in Italy (LMG 26824), and was kindly donated by Dr. Paola Mercuri (Ulg). Previous studies were focusing on the changes in the intracellular and membrane protein composition upon treatment of this strain with β-lactam antibiotics (Van Oudenhove et al., 2012). This revealed a β-lactam-induced upregulation of two Ax21 homologues. Ax21 is an outer membrane protein involved in virulence and biofilm formation, and is known to be associated with outer membrane vesicles (OMVs) in related organisms (Bahar et al., 2014). Several studies report on the function of OMVs in the response of bacteria against antibiotics. For example, β-lactam-induced vesiculation was already demonstrated in different species and it was proven that these vesicles contain β-lactamase enzymes (Ciofu et al., 2000; Schaar et al., 2011; Lee et al., 2013; Stentz et al., 2015).

The first aim of this research was to study the effect of the β-lactam imipenem on *S. maltophilia* OMV secretion, by determining the OMV concentration, morphology, and protein cargo. Next to imipenem, diffusible signal factor (DSF) quorum sensing molecules from *S. maltophilia*, and from *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*, were used as well to study intra- and inter-species effects on OMV secretion. Finally, the influence of these stimuli were analyzed on the expression and subsequent OMV mediated secretion of the Ax21 virulence factor. These findings are documented in chapter 3.

Proteomic characterization of the *S. maltophilia* OMVs indeed showed the presence of the L1 and L2 β-lactamases inside the OMVs. Therefore the β-lactamase activity of intact OMVs was assayed, and consequently we evaluated the potential of these OMVs to degrade clinically relevant β-lactam antibiotics. The potential biological impact of β-lactamase carrying OMVs on polymicrobial communities was explored by looking at changes in antibiotic tolerance in *S. maltophilia*, *P. aeruginosa* and *B. cenocepacia* cultures. Besides direct antibiotic degradation by the OMVs, the vesicles may also influence biofilm formation. Biofilm assays were performed to investigate the effect of OMVs on biofilm formation on the aforementioned species. The research concerning the role of OMVs in antibiotic resistance is described in chapter 4.

Next to β-lactam antibiotics, also other types of antibiotics may potentially induce vesiculation. In *P. aeruginosa* the fluoroquinolone ciprofloxacin leads to increased vesiculation through the induction of the SOS response (Maredia et al., 2012). Vesicles from ciprofloxacin-exposed *S. maltophilia* cultures were characterized and compared to imipenem-induced vesicles. Observed differences were investigated further into detail to generate insights about the potential impact of the ciprofloxacin-triggered vesiculation response.

The proposed goals will contribute to a broader knowledge on antibiotic resistance in *S. maltophilia* and beyond, and will help us to gain perspective on the role of this species in polymicrobial infections. On the other hand, understanding how bacterial membrane vesicles are produced, how the beneficial or harmfull vesicle cargo is selected, and how the vesicles establish cell interactions, could lead to new or improved membrane vesicle-based treatments of bacterial infections.

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Experimental work

- Chapter 3 -

Characterization of *Stenotrophomonas maltophilia* outer membrane vesicles

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This work was published as 'The effect of imipenem and diffusible signaling factors on the secretion of outer membrane vesicles and associated Ax21 proteins in *Stenotrophomonas maltophilia*' by Devos S., L. Van Oudenhove, S. Stremersch, W. Van Putte, R. De Rycke, G. Van Driessche, J. Vitse, K. Raemdonck and B. Devreese. Front Microbiol (2015) 6, 298.

Author contributions

I prepared the OMVs, performed the 2D-LCMS^{E} OMV profiling experiment, as well as the LC-MRM analysis, and wrote the manuscript. LV performed the time-kinetic, quantitative proteome study. GV provided technical support for all measurements with the NanoAcquity UPLC® system and the SYNAPT™ G1 HDMS mass spectrometer. SS carried out the OMV quantification experiments, with help of JV, and supervision of KR. WV did the TEM imaging of OMVs, under supervision of RD. SD drafted the manuscript. BD supervised the research and edited the manuscript.

Abstract

Outer membrane vesicles (OMVs) are small nanoscale structures that are secreted by bacteria and that can carry nucleic acids, proteins and small metabolites. They can mediate intracellular communication and play a role in virulence. In this study, we show that treatment with the β-lactam antibiotic imipenem leads to a dramatic increase in the secretion of outer membrane vesicles in the nosocomial pathogen *Stenotrophomonas maltophilia*. Proteomic analysis of their protein content demonstrated that the OMVs contain the chromosomal encoded L1 metallo-β-lactamase and L2 serine-β-lactamase. Moreover, the secreted OMVs contain large amounts of two Ax21 homologues, i.e. outer membrane proteins known to be involved in virulence and biofilm formation. We show that OMV secretion and the levels of Ax21 in the OMVs are dependent on the quorum sensing diffusible signal system (DSF). More specific, we demonstrate that the *S. maltophilia* DSF cis-Δ2-11-methyl-dodecenoic acid and, to a lesser extent, the *Burkholderia cenocepacia* DSF cis-Δ2-dodecenoic acid, stimulate OMV secretion. By a targeted proteomic analysis, we confirmed that DSF-induced OMVs contain large amounts of the Ax21 homologues, but not the β-lactamases. This work illustrates that both quorum sensing and disturbance of the peptidoglycan biosynthesis provoke the release of OMVs and that OMV content is context dependent.

Keywords

Outer membrane vesicles, *Stenotrophomonas maltophilia*, antibiotic resistance, proteomics, quorum sensing

3.1 Introduction

Stenotrophomonas maltophilia is the most frequently isolated unusual non-fermenting Gram-negative bacterium in hospitalized patients (Fihman et al., 2012). It is associated with an expanding range of clinical syndromes like bacteraemia, pneumonia and soft-tissue infections. *S. maltophilia* is also one of the most common emerging multi-drug resistant organisms found in the lungs of cystic fibrosis (CF) patients, with increasing prevalence (Amin and Waters, 2014). The high mortality in immuno-compromised patients observed with these infections is associated with the intrinsic and acquired resistance of *S. maltophilia* to many of the currently used antibiotics, including those of the β-lactam carbapenem type (Fihman et al., 2012). Indeed, antibiotic stress induces the production of two different chromosomal encoded carbapenem-hydrolyzing β-lactamases, the L1 metallo- and L2 serine-β-lactamases (Van Oudenhove et al., 2012).

In some species, chromosomal encoded β-lactamases can be secreted in outer membrane vesicles (OMVs), enabling extracellular β-lactam degradation (Ciofu et al., 2000). Such OMVs are actually secreted by all Gram-negative bacteria and have different biological functions including protection of the secreted cargo, long-distance transport of toxins and virulence factors, cell-cell communication, pathogenesis, antibiotic resistance and aiding in biofilm formation (Deatherage et al., 2009; Bonnington and Kuehn, 2013; Tiwari, 2014). Recently, Ax21 was identified in *S. maltophilia* as an important OMV-associated virulence factor (McCarthy et al., 2011; Ferrer-Navarro et al., 2013). While the actual function of the Ax21 protein in *S. maltophilia* is still uncertain, studies in the closely related plant pathogen *Xanthomonas oryzae* have provided evidence for a role in motility and biofilm formation (Park et al., 2014). *Xanthomonas* Ax21, recently renamed to omp1X, is an outer membrane beta barrel protein that is secreted by the general secretion (Sec) system, and it is associated with outer membrane vesicles (OMVs) as well (Bahar et al., 2014).

In *X. oryzae* pv. *oryzicola*, Ax21 expression was found to be regulated by the diffusible signal factor (DSF) quorum sensing (QS) system (Qian et al., 2013). The DSF system is a well-established QS system involved in cell-cell signaling and the regulation of biofilm formation in *S. maltophilia* (Fouhy et al., 2007; Ryan and Dow, 2010). DSF cell-cell signaling is managed by 'regulation of pathogenicity factors' (rpf) proteins, encoded in the *rpfBFCG* gene cluster (Huedo et al., 2014). The enoyl-CoA hydratase family enzyme RpfF and the long-chain fatty acyl coenzyme A ligase RpfB are responsible for the synthesis of cis-Δ2-11-methyl-dodecenoic acid, the main DSF molecule in *S. maltophilia* (Huang and Lee Wong, 2007). Extracellular DSF can be perceived by the two-component system (TCS) histidine sensor kinase RpfC, which activates the RpfG response regulator. The activated RpfG then acts as a cyclic diguanosine monophosphate (c-di-GMP) phosphodiesterase, influencing cellular c-di-GMP levels and downstream regulation events (Ryan and Dow, 2010; Tao et al., 2010). Interestingly, the *S. maltophilia* DSF molecule can also influence other species like *Pseudomonas aeruginosa* and *Burkholderia cenocepacia* (Ryan and Dow, 2010), which often co-colonize in the lungs of patients with cystic fibrosis (CF) (Twomey et al., 2012).

It is still unknown what mechanisms are responsible for OMV biogenesis, and how its content is selected. However, it is believed that it entails an active process, and based on the OMV cargo, several models have been proposed (Haurat et al., 2014). A recent study on another species within the Xanthomonadaceae, the plant pathogen *Xylella fastidiosa*, showed that OMV biogenesis (and virulence) is suppressed by the DSF system (Ionescu et al., 2014). The aim of this study was to investigate the effect of different DSFs on the secretion of OMVs in *S. maltophilia*, as well as the influence of β-lactam antibiotic treatment. We also report on the OMV-associated secretion of two Ax21 homologues and on how DSFs affect the abundance of these homologues in OMVs.

3.2 Material and methods

3.2.1 Materials

Urea was obtained from GE Healthcare (Diegem, Belgium). Tris-HCl (UltraPure™, 1M, pH 8) was purchased from Invitrogen (Carlsbad, CA, US). Bovine serum albumin (MS grade protein standard) was purchased from Protea Biosciences Group (Morgantown, WV, US). Sequencing grade modified trypsin was obtained from Promega (Madison, WI, US). ULC-MS grade water, acetonitrile (ACN) and formic acid was procured from Biosolve (Valkenswaard, The Netherlands). Imipenem was kindly donated by Prof. M. Galleni (CIP, University of Liège, Belgium). Other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, US).

3.2.2 Bacterial cell culture

The imipenem-resistant *S. maltophilia* strain 44/98 (LMG 26824, a kind gift of Dr. Paola Mercuri, Ulg, Belgium) was isolated at the Clinical Microbiology Unit of the Varese University hospital in Italy. Cultures were grown aerobically overnight in Luria Broth (LB) until the stationary phase. The cell suspensions were then diluted to an OD_{600nm} of 0.2, grown until the mid-exponential growth phase (OD_{600nm} = 0.65-0.75), and then stimulated with either 25 µg/mL imipenem, 1 mM *Stenotrophomonas maltophilia* DSF (cis-Δ2-11-methyl-dodecenoic acid), 1 mM *Burkholderia cenocepacia* DSF (BDSF, cis-Δ2-dodecenoic acid), or 1 mM *Pseudomonas aeruginosa* DSF (PDSF, cis-Δ2-decenoic acid), and allowed to grow further for 3 h.

3.2.3 Time-kinetic, quantitative proteome study

Methodology is provided in Addendum A1.

3.2.4 Isolation of outer membrane vesicles

To obtain cell free culture supernatant from *S. maltophilia*, cells were pelleted by centrifugation at 6,000 x g for 5 min, and the culture supernatant was filtered through a 0.2 µm V25 vacuum filter (Sarstedt, Numbrecht, GE). The OMVs were pelleted by ultracentrifugation at 100,000 x g for 1 h (Avanti J-30I, Beckman Coulter, Fullerton, CA). One milliliter of the filtered culture supernatant was spread onto an LB agar plate and incubated at 37°C for 24 h to confirm the absence of intact, living cells.

3.2.5 OMV protein extraction and digestion

OMV proteins were extracted by dissolving the OMV pellet in 1 ml 8 M urea in 50 mM Tris-HCl pH 8. Proteins were precipitated with trichloroacetic acid (20%) and consequently the pellet was washed twice with ice-cold acetone, and finally dissolved in 2 M urea in 50 mM ammonium bicarbonate (pH 8). The protein concentration was assessed using the Coomassie Plus Bradford™ Assay kit (Thermo Scientific, San Jose, CA, US). Proteins were reduced with 10 mM dithiothreitol for 30 min at 60 °C, alkylated with 20 mM iodoacetamide at ambient temperature for 30 min, and then digested with trypsin (1:50 w/w) overnight at 37 °C.

3.2.6 LCMS^E analysis of outer membrane vesicle content

Peptide mixtures (0.5 µg/µl in 100 mM ammonium formate, pH 10) were separated on a NanoAcquity UPLC® system (Waters Corporation, Milford, MA) in 2D mode. For the first dimension (high pH), solvent A1 and B1 were composed of 20 mM ammonium formate in water and ACN (pH 10), respectively. For the second dimension (low pH), solvent A2 and B2 were composed of 0.1% formic acid in water and 0.1% formic acid in ACN, respectively. The sample (1 µg) was loaded onto an Xbridge™ BEH130 C18 column (300 µm x 50 mm, 5 µm; Waters) at 3% solvent B1 at 2 µL/min. Peptides were eluted from the first dimension column in 5 fractions (11.1%, 14.5%, 17.4%, 20.8% and 45.0% of solvent B1), and fractions were trapped on a

Symmetry® C18 trapping column (180 µm x 20 mm, 5 µm; Waters). Each fraction was separated on a HSS T3 C18 analytical column (75 µm x 250 mm, 1.8 µm; Waters) at 40°C at 250 nL/min by increasing the acetonitrile concentration from 5-50% B2 over 60 min.

The outlet of the column was directly connected to a PicoTip Emitter (uncoated SilicaTip™ 10 +/- 1 µm, New Objective, Woburn, MA, US) mounted on a Nanolockspray source of a SYNAPT™ G1 HDMS mass spectrometer (Waters). The time-of-flight (TOF) analyzer was externally calibrated with MS/MS fragments of human [glu^1]-fibrinopeptide B (Glu-fib) from *m/z* 72 to 1285, and the data was corrected post-acquisition using the monoisotopic mass of the doubly charged precursor of Glu-fib (*m/z* 785.8426) (lock mass correction). Accurate mass data were collected in a data independent positive mode of acquisition (MS^E) by alternating between low (5 V) and high (ramping from 15 to 35 V) energy scan functions (Geromanos et al., 2009). The selected *m/z* range was 125 to 2000 Da. The capillary voltage was set to 3.0 kV, the sampling cone voltage was 26 V and the extraction cone voltage on 2.65 V. The source temperature was set on 65 °C.

The LCMS^E data were processed using the ProteinLynx Global SERVER™ v2.5 (PLGS, Waters Corporation) (Geromanos et al., 2009). In brief, lock mass-corrected spectra (0.250 Da window allowed) were automatically centroided, deisotoped and charge-state reduced to produce a single monoisotopic peak for each peptide and associated fragment ion. The correlation of a precursor and a potential fragment ion was achieved by means of time alignment. The following parameters were used for the data processing in PLGS: the chromatographic peak width, the TOF resolution and retention time window, which were determined automatically by the software, and the low energy, high energy, and intensity thresholds, which were set to 250, 100 and 1500 counts respectively. A database containing 4380 protein entries from the closely related *Stenotrophomonas maltophilia* K279a (downloaded from the Uniprot website, April 2014), together with a decoy database consisting of the randomized entries of all the proteins, was interrogated by PLGS (Li et al., 2009). The precursor and fragment ion tolerance were determined automatically. The default protein identification criteria used included a maximal protein mass of 250,000 Da, a detection of minimal 3 fragment ions per peptide, minimal 7 fragment ions per

protein and minimal 1 peptide per protein. Carbamidomethyl-C (fixed) and methionine oxidation (variable) were selected as modifications. Maximally one missed cleavages and a false positive rate of 4% was allowed.

3.2.7 Sample preparation for liquid chromatography-multiple reaction monitoring (LC-MRM) analysis

Cells, obtained from 10 ml of culture, were pelleted by centrifugation at 6,000xg for 5 min, and the culture supernatant was filtered through a syringe-driven 0.22 µm PES membrane filter unit (Merck Millipore, Darmstadt, GE). OMV proteins were isolated as described above, and finally dissolved in 50 µl 2 M urea in 50 mM ammonium bicarbonate. Protein solutions were spiked with 100 ng BSA (MS grade protein standard), reduced and alkylated, and digested with 0.5 µg trypsin. Digested samples were dried and dissolved in 50 µl 0.1% formic acid in water for LC-MRM analysis (5 µl injection). This procedure was followed for 2 biological replicates.

3.2.8 LC-MRM analysis

The digested samples were first separated by RPLC on a U3000-RSLC system (Thermo). Briefly, the sample was loaded onto an Acclaim PepMap100 pre-concentration column (L x ID 2 cm x 100 µm, C18, 5 µm, 100Å) at a flow rate of 5 µl/min, and flushed for 3 min with 0.1% HCOOH / 2% ACN. The sample was then separated on a Thermo Acclaim PepMap100 analytical column (L x ID 15 cm x 75 µm, C18, 3 µm, 100Å) at a flow rate of 300 nl/min, with mobile phases 0.1% HCOOH in water (solvent A) and 0.1% HCOOH in ACN (solvent B). Peptides were separated with a 30 min gradient, going from 2% to 40% solvent B, and eluting peptides were sprayed directly in a 4000 QTRAP mass spectrometer (AB Sciex, Framingham, MA) with a NanoSpray II ESI source (AB Sciex), using a PicoTip Emitter (uncoated SilicaTip^{TM} 10 +/- 1 µm). The ion spray voltage was set at 3.5 kV, curtain gas at 10 (arbitrary units), nebulizing gas at 5, and interface heater temperature at 60°C. We analysed 2 technical replicates per biological samples.

Target peptides (two for each protein) were measured in multiple reaction monitoring (MRM) acquisition mode, with the Q1/Q3 resolution set at LOW and with a maximum total cycle time of 3 sec. The double charged peptide was selected as precursor (Q1), and for each precursor three fragment ions were selected from the y-ions (Q3). The collision energy (CE) and declustering potential (DP) were calculated with the following equations:

CE (V) = (0.5 x m/z) + 5

DP (V) = (0.0729 x m/z) + 31.117

The MRM data was imported in Skyline v2.5 (MacLean et al., 2010) and peak traces were subjected to a Savitzky-Golay Smoothing transformation. The total area under curve (AUC) of each target peptide was calculated, and normalized to the spiked BSA standard. A student's t-test was performed to evaluate the significance of differential protein abundancy levels between stimulated and unstimulated cultures.

3.2.9 OMV quantification

OMVs from 25 ml cultures were harvested as described above, and the pellet was dissolved in 100 µl phosphate buffered saline (PBS). The OMV concentration and size was determined by light scattering based single particle tracking using a NanoSight LM10-HS instrument (NanoSight, Amesbury, UK; Dr. A. Hendrix, Laboratory of Experimental Cancer Research, Ghent University Hospital) equipped with a 405 nm laser. Prior to analysis, the purified OMVs were diluted in PBS-buffer (Invitrogen). Movies of 60 seconds were recorded and analyzed with the NTA Analytical Software version 2.3. Each individual sample was diluted and measured 3 times. Calculations were performed according to Van der Pol et al. (2010).

3.2.10 Transmission electron microscopy

The *S. maltophilia* strain 44/98 was cultured overnight, as described above. At the mid exponential growth phase, cultures were diluted in LB medium supplemented with 25 µg/mL imipenem, and allowed to grow further for 2 h. A control culture was treated similarly except for the addition of imipenem. A 4 µL drop of culture was placed on a Formvar/carbon-coated cupper grid, made hydrophilic by glow discharging for 30 s. The grid was then washed by placing it sequentially onto 5 drops of milliQ water. After these washing steps, the grid was placed on 2 drops of 2% uranyl acetate and incubated for 30 to 40 s. The grid was blotted with filter paper between each washing step. The created specimens were examined using a JEM 1010 transmission electron microscope (JEOL, Tokyo, Japan) operating at 60 kV using Image Plate Technology from Ditabis (Pforzheim, Germany).

3.3 Results

3.3.1 Imipenem stimulates the expression of two Ax21 homologues

A preliminary time-kinetic, quantitative proteome study on the imipenem response of *S. maltophilia* was performed to address the proteome dynamics after antibiotic treatment. Among the few proteins of which we found a gradual increase in abundancy over time after exposure to imipenem, we found mainly the two β-lactamases, outer membrane proteins and proteins involved in motility (flagellins). Two of these proteins showed strong homology to *X. oryzae* Ax21 (PXO_03968), i.e. Smlt0387 and Smlt0184 (Figure 9, A). Former studies on Ax21 in *S. maltophilia* report only a single Ax21 homologue (Smlt0387) (McCarthy et al., 2011; Ferrer-Navarro et al., 2013). We found now thus two homologues with respectively 60% identity for Smlt0387 (e-value 7.0e-80) and 56% identity for Smlt0184 (e-value 4.0e-73) with the *X. oryzae* protein. Alignment of Smlt0387 and Smlt0184 learned that the two proteins display 63% identity. The alignment of the two *S. maltophilia* Ax21 sequences with the *X. oryzae* Ax21 sequence is depicted in Figure 9B. Both proteins contain a typical signal sequence (Figure 9, B; green box) implicating that, like the *X. oryzae* Ax21 protein, they are processed by the Sec secretion system (Bahar et al., 2014). Analyzing the protein sequences of the *S. maltophilia* Ax21 homologues Smlt0387 and Smlt0184 with Pfam (Finn et al., 2014) showed that they belong to the outer membrane protein β-barrel domain family, with e-values 1.5e-09 and 5.7e-11 respectively. This is consistent with the structural model, representing a porin-like structure, proposed recently (Park et al., 2014).

3.3.2 Imipenem-treated *Stenotrophomonas maltophilia* cells secrete more OMVs

A recent study demonstrated that the *X. oryzae* Ax21 protein is secreted in association with OMVs (Bahar et al., 2014). Therefore, we verified the production of OMVs by *S. maltophilia* upon imipenem stress. As a matter of fact, it was shown in *Acinetobacter baumanii* (ATCC19606^T) that exposure to subinhibitory concentrations of ceftazidime (cephalosporine, also a β-lactam) caused ruffling along the whole outer membrane resulting in the formation of more OMVs (Koning et al.,

2013). Using TEM, we indeed observed a similar increase in OMV secretion in *S. maltophilia,* when exposed to imipenem (Figure 10, A-B). When we applied light scattering based single particle tracking to quantify the isolated OMVs from an equal volume of stimulated and unstimulated cultures (25 ml culture, 3h imipenem stimulation), the stimulated cultures contained considerable more OMVs than the unstimulated cultures (Figure 10, C).

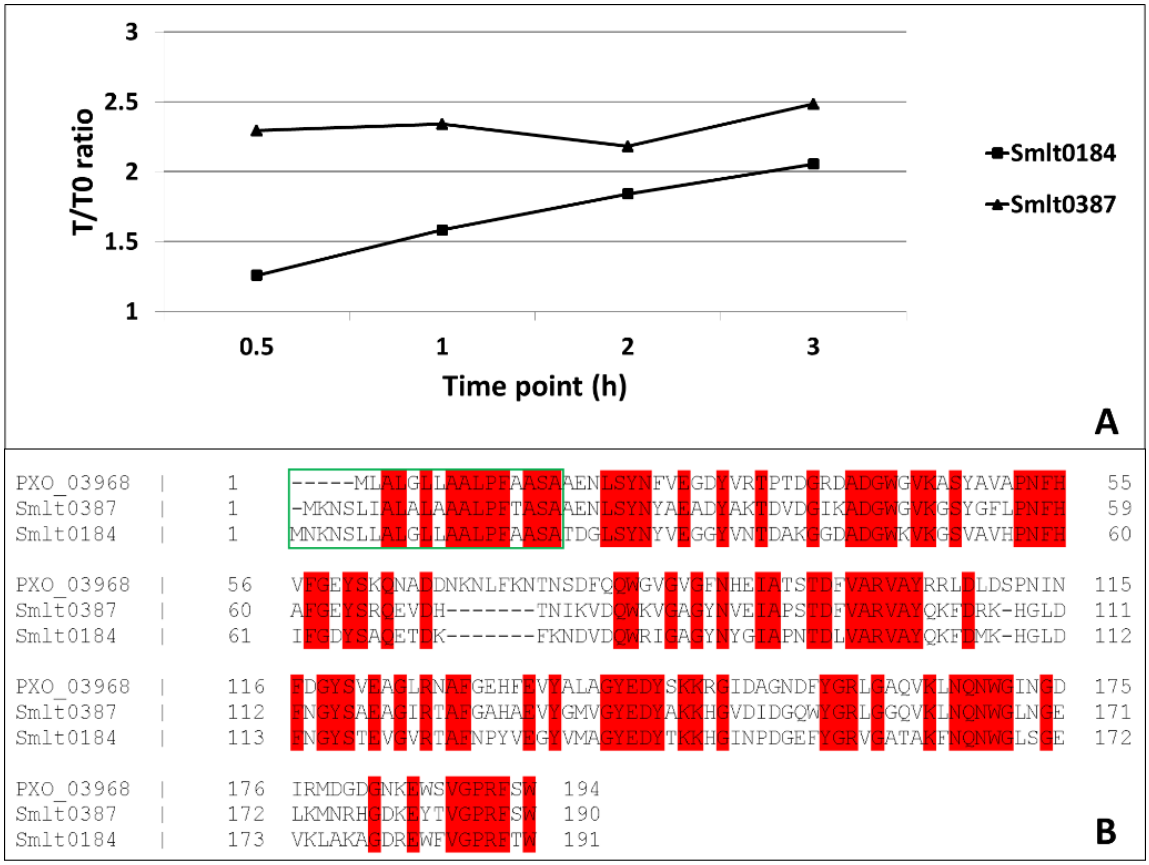

Figure 9. (**A**) Time-dependent increase in abundancy of the two *S. maltophilia* Ax21 homologues (Smlt0387 and Smlt0184). For each time point, the abundance ratio with reference to time point 0 is plotted. (**B**) Alignment of Smlt0387 and Smlt0184 with the *Xanthomonas* Ax21 protein (PXO_03968). Red: identical amino acid residues. Green box: signal sequence.

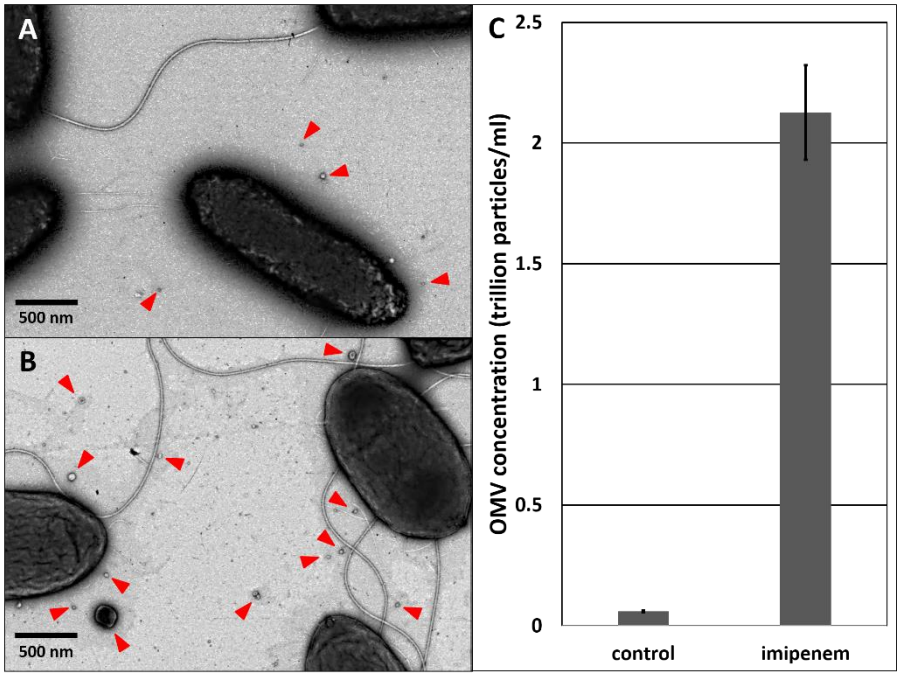

Figure 10. OMV visualization with TEM: (**A**) control, (**B**) after imipenem treatment. OMV concentration determination with light scattering based single particle tracking after imipenem treatment (**C**). Error bars plot the standard deviation.

3.3.3 Proteomic analysis of *S. maltophilia* OMVs

To confirm the presence of both Ax21 proteins in OMVs, a profile of the *S. maltophilia* OMV proteome was assessed with LCMS^E. Cells were grown in the presence of the broad-spectrum β-lactam antibiotic imipenem (3h). After collection of the OMVs and protein extraction, one microgram of digested protein was separated with 2D-UPLC (high pH – low pH RPLC) on a NanoAcquity UPLC® system, and eluting peptides were analyzed online with a SYNAPT™ HDMS Q-TOF mass spectrometer. The acquisition of MS/MS spectra in a data-independent acquisition mode (MS^E) resulted in the identification of 234 proteins (identified in at least two of the three technical replicate LCMS^E runs) (Addendum A2). Indeed, the Ax21 proteins Smlt0387 and Smlt0184 were identified in all three technical replicate runs with, on average, a sequence coverage of 82.1% and 70.0 % respectively. These findings confirm the secretion of Ax21 as an outer membrane protein associated with outer membrane vesicles in *S. maltophilia*.

The proteins identified in the OMVs were further annotated with Blast2GO v.2.7.1 and PSORTb v.3.0.2. Several proteins were identified that are involved in the assembly of β-barrel proteins: SecA and SecB for protein translocation to the periplasm, the SurA chaperone which prevents misfolding in the periplasm, and BamA, BamB, BamD and BamE, responsible for β-barrel protein assembly in the outer membrane (Selkrig et al., 2013). Apart from the Ax21 homologues, several other outer membrane proteins were identified, for example TonB-dependent receptor proteins, Omp family proteins, autotransporters, lipoproteins, and the SmeX efflux protein (part of the Resistance-Nodulation-Cell Division (RND) SmeVWX efflux pump). During OMV formation, periplasmic proteins can be encapsulated, as well as inner membrane (associated) proteins. This is illustrated by the identification of proteins involved in peptidoglycan turnover (penicillin-binding proteins (PBPs), carboxypeptidases, transglycosylases). Furthermore, fimbrial adhesins and flagellins were identified, involved in adhesion and motility, respectively. Interestingly, the OMVs also contain the L1-metallo- and L2-serine-β-lactamases (Smlt2667 and Smlt3722) when exposed to imipenem. Both β-lactamases are translocated to the periplasm via different systems: the L1-β-lactamase uses the Sec export system, the L2-β-lactamase uses the Tat export system (Pradel et al., 2009; Brooke, 2012). Finally, the OMV proteome profile also includes cytoplasmic proteins, mostly highly abundant proteins (e.g. elongation factors) and ribosomal proteins.

3.3.4 Influence of diffusible signaling factors on the secretion of OMVs

Next to the β-lactam antibiotic imipenem, also DSF quorum sensing molecules were tested for their ability to stimulate OMV secretion (Figure 11, A). A study on the plant pathogen *Xylella fastidiosa* showed a link between OMV secretion and the DSF quorum sensing system (Ionescu et al., 2014). The authors postulated that OMVs are affecting plant colonization by blocking surfaces, leading to a deeper spread of *X. fastidiosa* into the plant host, which increases virulence. The *X. fastidiosa* DSF system suppresses the release of OMVs (and virulence), causing cells to grow more locally, attached to unblocked surfaces. However, in *S. maltophilia*, growing cells in presence of its own DSF cis-Δ2-11-methyl-dodecenoic acid resulted in a remarkable increase in OMV secretion (Figure 11, B), comparable to the amount secreted in the presence of imipenem. The DSF cis-Δ2-

dodecenoic acid produced by *B. cenocepacia* (BDSF) also led to a slight increase in OMV secretion in *S. maltophilia*, while the DSF cis-Δ2-decenoic acid produced by *P. aeruginosa* (PDSF) did not. This is in accordance with the known responsiveness of *S. maltophilia* to these DSF molecules. *S. maltophilia* can perceive the DSFs produced by itself and *B. cenocepacia*, but not the one produced by *P. aeruginosa* (Ryan and Dow, 2010). The endogenous methyl-branched DSF was shown to be far more active than the unbranched BDSF and PDSF.

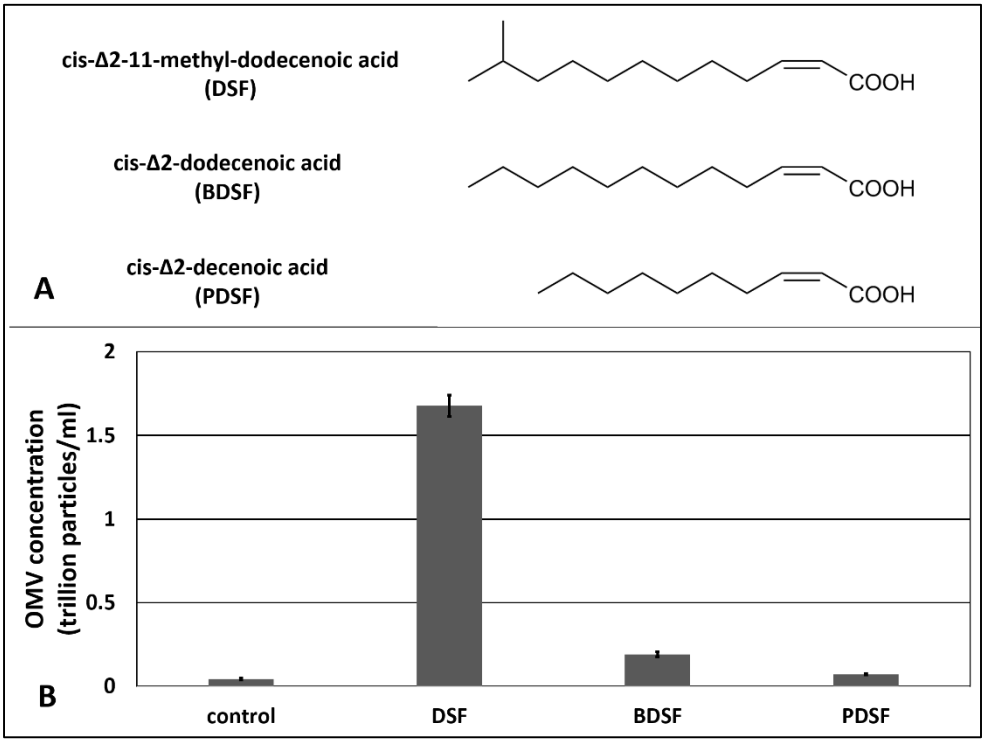

Figure 11. (**A**) Structure of DSF, BDSF and PDSF. (**B**) Effect of DSF and its structural homologues on OMV secretion determined with light scattering based single particle tracking. Error bars plot the standard deviation.

3.3.5 Relative quantification of OMV-associated proteins with multiple reaction monitoring (MRM)

The differential OMV-associated secretion of the Ax21 homologues was validated with a label-free, targeted LC-MRM approach. Two proteotypic peptides for each Ax21 protein were quantitatively analysed with MRM: (K-)VGAGYNVEIAPSTDFVAR(-V) and (K-)LNQNWGLNGELK(-M) for Smlt0387, and (R-)IGAGYNYGIAPNTDLVAR(-V) and (K-)FNQNWGLSGEVK(-L) for Smlt0184. Three MS/MS transitions for each peptide were chosen (Addendum A3). The peptides are unique within the *S. maltophilia* K279a proteome, and a UniPept search (Mesuere et al., 2012) also revealed uniqueness for *S. maltophilia* species. These peptides also show good MRM compatibility in terms of length, hydrophobicity, and ionization properties. In addition, the dominant L1 β-lactamase (Smlt2667) (target peptides GVAPQDLR and IAYADSLSAPGYQLK) and the spiked-in BSA standard (target peptides AEFVEVTK and QTALVELLK) were monitored. The MRM analysis was performed on OMV protein extracts from equal culture volumes grown in presence of imipenem, DSF, BDSF and PDSF (3h stimulation).

These experiments confirmed a huge increase in OMV-associated secretion of both Ax21 proteins, when *S. maltophilia* cultures were stimulated with the β-lactam antibiotic imipenem, and with the DSF and BDSF quorum sensing molecules (Figure 12, A-B). When comparing the two Ax21 homologues, the previously overlooked homologue Smlt0184 is actually much more prevalent than Smlt0387, both in the imipenem-stimulated culture, as well as in the DSF- and BDSF-stimulated cultures. Also remarkable is that the highest amount of Smlt0184 was measured in the BDSF-induced OMVs (Figure 12, A), while the OMV production elicited by BDSF is in fact much lower than for imipenem and DSF (Figure 11, B). Smlt0387 secretion seems to be more pronounced after imipenem exposure, than it is for DSF or BDSF (Figure 12, B). *S. maltophilia* is again unresponsive to the *P. aeruginosa* PDSF in terms of Ax21 secretion, as it was for OMV production (Figure 12, B).

The large increase in L1 β-lactamase expression in response to imipenem is also represented here in the secreted OMVs (Figure 12, C), as previously observed at the cellular level (Van Oudenhove et al., 2012). The fast production of β-lactamases, especially the L1 metallo-β-lactamase, represents the important early line of defense of *S. maltophilia* against the imipenem challenge. Since imipenem leads to substantial L1 levels in the cell, and the periplasm, it is not entirely surprising that this protein is also prevalent in the secreted OMVs. Nevertheless, whether its presence in the OMVs is accidental or predestined, its biological relevance is obvious. Finally, only a slight increase in OMV-associated L1 secretion was observed when stimulated with DSF, and even more with BDSF (Figure 12, C).

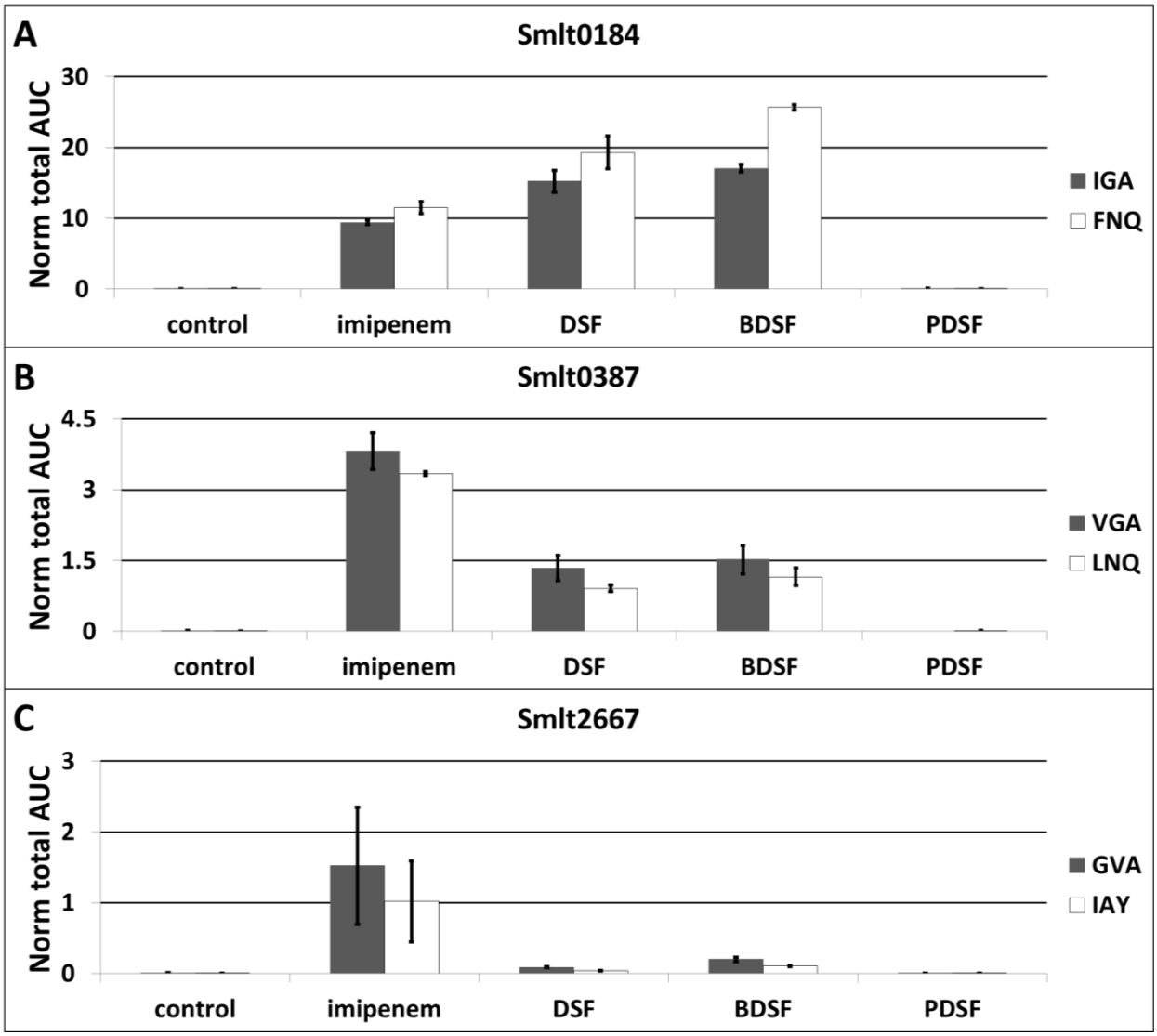

Figure 12. Relative abundancy of OMV-associated Ax21 homologues (**A-B**) and L1 β-lactamase (**C**) in response to imipenem and diffusible signaling factors, determined by targeted proteomics (LC-MRM). The plot display the average normalized area-under-the-curve for each peptide used as marker for the different proteins. The error bars plot the standard deviation. Target peptides: VGA: VGAGYNVEIAPSTDFVAR, LNQ: LNQNWGLNGELK, IGA: IGAGYNYGIAPNTDLVAR, FNQ: FNQNWGLSGEVK, GVA: GVAPQDLR, IAY: IAYADSLSAPGYQLK.

3.4 Discussion

The genome sequence of the pathogenic *Stenotrophomonas maltophilia* K279a strain revealed an organism that is well adjusted for living in an environment with antibiotics (Crossman et al., 2008). We here add the capacity of *S. maltophilia* to secrete OMVs packed with β-lactamases as an additional property to adapt to antibiotic stress.

In this work, we quantified the amount of OMV secretion as a response to the β-lactam antibiotic imipenem. As expected, imipenem led to a significant increase in OMV secretion, probably owing to the disturbed cell wall structure or alteration in peptidoglycan dynamics (Haurat et al., 2014). Proteomic analysis on the isolated OMVs revealed the OMV-mediated secretion of the chromosomal encoded β-lactamases in *S. maltophilia*. This is not the case when OMVs are induced by DSF. Whether the presence of β-lactamases in the OMVs is merely due to their high abundance in the periplasm upon β-lactam stress or whether they are deliberately delivered in the OMVs is not clear. Anyway, by exporting the β-lactamases in the environment, *S. maltophilia* not only provides resistance against the imipenem at the cell level, but could also protect other cells from the same species, or from other species. Additionally, packed in OMVs, the β-lactamases are protected against extracellular degradative enzymes and are able to travel long distances (Bonnington and Kuehn, 2013).

In *Xylella fastidiosa*, the production of OMVs is suppressed by the DSF quorum sensing system (Ionescu et al., 2014). Therefore, the DSF quorum sensing molecules cis-Δ2-11-methyl-dodecenoic acid (DSF), cis-Δ2-dodecenoic acid (BDSF) and cis-Δ2-decenoic acid (PDSF), produced by *S. maltophilia*, *B. cenocepacia* and *P. aeruginosa* respectively, were also tested for their effect on OMV production in *S. maltophilia*. In contrast to *X. fastidiosa*, DSF led to a comparable increase in the amount of OMV secretion as with imipenem. BDSF led to a slight increase in OMV secretion, and PDSF did not have any effect. These fatty acid analogues do not have any known perturbation effects on the cell wall, as opposed to imipenem, and the OMV response is in agreement with

their signaling activity in *S. maltophilia*. These results therefore suggest a quorum sensing controlled OMV biogenesis.

Finally, the OMV proteome analysis revealed the production of two Ax21 proteins in *S. maltophilia*. The Ax21 protein was shown to be involved in biofilm formation and virulence, and is highly conserved in all *Xanthomonas* species, and in *S. maltophilia*. Recently, it was shown that the Xanthomonas Ax21 is an outer membrane protein, secreted in OMVs (Bahar et al., 2014), and regulation of expression is dependent on the DSF system (Qian et al., 2013). With a targeted and label-free MRM method, we quantified the OMV-mediated secretion of the Ax21 homologues as a response to imipenem, DSF, BDSF and PDSF. All conditions led to substantial amounts of OMV-associated Ax21 secretion. The results indicate a deliberate and regulated secretion of Ax21, rather than a coincidental presence due to an increased OMV biogenesis. The production of OMVs and packing with large quantities of Ax21 protein, point to an important role of it in antibiotic resistance and biofilm formation. However, the exact role of Ax21 in *S. maltophilia* remains unclear, and should be further investigated, more specific by confirming its porin transporter function and determination of its cargo.

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- Chapter 4 -

Intra- and inter-species effect of outer membrane vesicles from *Stenotrophomonas maltophilia* on resistance and biofilm formation

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Part of this work is adapted for publication as ‘Intra- and inter-species effect of outer membrane vesicles from *Stenotrophomonas maltophilia* on β-lactam resistance‘ by Devos S., S. Stremersch, K. Raemdonck, K. Braeckmans and B. Devreese (Antimicrobial Agents and Chemotherapy, 2016 - Accepted)

Author contributions

I performed all experiments, with the exception of the OMV quantification experiment. SS carried out the OMV quantification experiment, with the supervision of KR and KB. I wrote the manuscript. BD supervised the research and edited the manuscript.

Abstract

The treatment of *Stenotrophomonas maltophilia* with β-lactam antibiotics leads to increased release of outer membrane vesicles (OMV), which are packed with two chromosomal encoded β-lactamases. Here we show that these β-lactamase-packed OMVs are capable of establishing extracellular β-lactam degradation. We also show that they dramatically increase the apparent MIC of imipenem and ticarcillin for co-habituating species *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. In addition to conferring OMV-mediated resistance by direct inactivation of the β-lactam antibiotics, OMVs are also involved in initiating and maintaining biofilm growth. Although OMVs from *S. maltophilia* have a negative effect on biofilm formation of its own species, these same OMVs display a positive effect on biofilm formation of *P. aeruginosa* and *B. cenocepacia*.

Keywords

Membrane vesicles, antibiotic resistance, beta-lactamases, biofilms, cystic fibrosis

4.1 Introduction

The multidrug resistant bacterium *Stenotrophomonas maltophilia* exploits a variety of mechanisms to resist antibiotic threats, i.e. the active extrusion of antibiotics by efflux pumps, alteration of cell membrane permeability, shielding by growing as a biofilm, or direct enzymatic inactivation of the antibiotic compounds (Abbott et al., 2011). β-lactam antibiotics like imipenem (IPM), amoxicillin (AMX) and ticarcillin (TIC) are frequently used to treat Gram-negative bacterial infections. Unfortunately these compounds are often ineffective for *S. maltophilia* infections due to the presence of two chromosomal encoded β-lactamase genes encoding the L1 metallo-β-lactamase and the L2 serine-β-lactamase, of which the expression immediately increases after exposure (Brooke et al., 2011).

In a previous study we revealed a significant increase in the release of outer membrane vesicles (OMVs) by *S. maltophilia* upon exposure to the broad spectrum carbapenem IPM (Chapter 3; Devos, Van Oudenhove et al., 2015). We showed that these vesicles are packed with L1 and L2 β-lactamases. OMVs are membranous nanostructures secreted by most Gram-negative bacteria. OMV secretion is typically higher under cellular stress conditions (Deatherage et al., 2009). The spherical outer membrane capsules enclose periplasmic material (e.g. proteins, peptidoglycan intermediates, signaling molecules) and transport them into the extracellular environment. This allows for long-distance transport and protection of cargo proteins (Bonnington and Keuhn, 2013). Several studies have shown the involvement of OMVs in antibiotic resistance (Ciofu et al., 2000; Schaar et al., 2011; Lee et al., 2013; Stentz et al., 2015). For example, OMVs have the potential to serve as vehicles for β-lactamases, allowing for the inactivation of β-lactam antibiotics before they can reach planktonic cells or biofilm communities.

OMVs have also been identified as important constituents of biofilm (Schooling and Beveridge, 2006). The formation of biofilm is an important property of *S. maltophilia*, as it is for many other pathogenic species, since it provides a protective environment against the host immune system and against antibiotic threats (Brooke et al., 2011; Jolivet-Gougeon and Bonnaure-Mallet, 2014;

de Oliveira-Garcia et al., 2003; Passerini de Rossi et al., 2007). In several species it was shown that OMVs influence cell aggregation, surface attachment and biofilm formation, both at inter- and intraspecies level. For example, OMVs produced by *Helicobacter pylori* enhance biofilm formation by establishing OMV-mediated cell-cell interactions (Yonezawa et al., 2009). OMVs from *Porphyromonas gingivalis* have a negative impact on biofilm formation of *Streptococcus gordonii* (Ho et al., 2015), but stimulate biofilm formation of *Staphylococcus aureus*, including methicillin-resistant *S. aureus* (MRSA) (Kamaguchi et al., 2003).

Multiresistant *S. maltophilia* is increasingly found in respiratory specimens from patients with cystic fibrosis (CF) (Raidt et al., 2015). The presence of *S. maltophilia* is a risk factor to generate a decline in the forced expiratory volume in 1 sec (FEV1) (Cogen et al., 2015). While the actual impact of this organism on CF disease is debated, its presence is of concern given its capacity to acquire antibiotic resistance (Looney et al., 2009; Sánchez, 2015). Considering that *S. maltophilia* co-colonizes with organisms like *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*, the presence of OMVs could potentially play an important role in multispecies biofilm initiation and maintenance, and thus biofilm-related resistance. Cell-cell communication is crucial for the survival of species in these communities, and interactions between the different species, whether synergistic or antagonistic, can influence the infection process, progress and treatment. The exposure of *S. maltophilia* inhabited communities to β-lactam antibiotics, and the subsequent secretion of β-lactamase-packed OMVs, could therefore potentially influence the effect of the β-lactams.

In this work, we explored the potential of β-lactam-induced OMVs to protect *S. maltophilia* cells, as well as *P. aeruginosa* and *B. cenocepacia* cells, against β-lactam treatment.

4.2 Material and methods

4.2.1 Materials

Nitrocefin was purchased from TOKU-E (Ghent, Belgium). ULC-MS grade water and acetonitrile (ACN) was procured from Biosolve (Valkenswaard, The Netherlands). Sequencing grade modified trypsin was obtained from Promega (Madison, WI, US). Acetic acid was purchased from VWR. All other chemicals and antibiotics were purchased from Sigma-Aldrich (St. Louis, MO, US).

4.2.2 Bacterial Cell Culture

The bacterial strains *Stenotrophomonas maltophilia* 44/98 (LMG 26824), *Pseudomonas aeruginosa* PAO1 (LMG 24986), and *Burkholderia cenocepacia* type strain (LMG 16656) were grown aerobically overnight in Luria Broth (LB) until the stationary phase. The cell suspensions were then diluted to an OD_{600nm} of 0.2, and allowed to grow further under the same conditions.

4.2.3 Preparation of outer membrane vesicles

S. maltophilia cells were grown for 2 hours until the mid-exponential growth phase (OD_{600nm} = 0.65-0.75), and then grown for an additional 3 hours in the presence of 1 mg/ml penicillin G (PEN). Cells were pelleted by centrifugation at 4,000 x g for 15 min. The culture supernatant was filtered through a syringe-driven 0.22 µm PES membrane filter unit (Merck Millipore, Darmstadt, GE), and the OMVs were pelleted by ultracentrifugation at 100,000 x g for 1 h (Avanti J-30I, Beckman Coulter, Fullerton, CA). The OMV pellet was resuspended in phosphate buffered saline (PBS) or LB. One milliliter of the filtered culture supernatant was spread onto an LB agar plate and incubated at 37°C for 24 h to confirm the absence of intact, living cells.

4.2.4 OMV quantification

Isolated OMVs from 50 ml cultures were harvested and resuspended in 1 ml PBS. The OMV concentration was determined by light scattering based single particle tracking using a NanoSight LM10-HS instrument (NanoSight, Amesbury, UK; Dr. A. Hendrix, Laboratory of Experimental Cancer Research, Ghent University Hospital) equipped with a 405 nm laser. Prior to analysis, the purified OMVs were diluted in PBS-buffer (Invitrogen). Movies of 60 seconds were recorded and analyzed with the NTA Analytical Software version 2.3. Each individual sample was diluted and measured 3 times. Calculations were performed according to Van der Pol et al. (2010).

Fluorescent single particle tracking measurements were used to determine the concentration of true membranous particles (Braeckmans et al., 2010). Prior to analysis, purified OMVs were fluorescently labeled with the membrane labeling dye PKH67 (Sigma-Aldrich). To this end OMVs were diluted 1 in 5 in diluent C and mixed with an equal volume of 140 µM PKH67 in diluent C. The mixture was incubated at 37 °C for 30 minutes and unincorporated dye was washed away by means of size exclusion chromatography using an exosome spin column (MWCO 3000) (Invitrogen) according to the manufacturer's instructions. Next, labeled OMVs were diluted in PBS and movies of the fast diffusing, individual vesicles were obtained at ambient temperature (22.5°C) using a swept field confocal microscope (LiveScan SFC, Nikon BeLux, Belgium) equipped with an Ixon Ultra EMCDD camera (Andor™ technologies), a 488 nm laser and a 60X oil immersion lens (NA 1.4; Nikon). Videos were analyzed with in-house developed software in Matlab® for particle detection, motion trajectory construction with calculation of diffusion coefficients and finally calculation of the OMV concentration according to a recently developed method with inherent calibration of the detection volume by Roding et al. (2011).

4.2.5 OMV proteomics

The OMV protein content from penicillin G stimulated *S. maltophilia* cultures was determined with a 2D-LCMS^E proteomics workflow as previously described (Chapter 3; Devos, Van Oudenhove

et al., 2015). Briefly, OMV proteins were reduced with 10 mM dithiothreitol for 30 min at 60 °C, alkylated with 20 mM iodoacetamide at ambient temperature for 30 min, and then digested with trypsin (1:50 w/w) overnight at 37 °C. Peptide mixtures were separated on a NanoAcquity UPLC® system (Waters Corporation, Milford, MA) in 2D mode. The LC outlet was directly connected to a PicoTip Emitter (uncoated SilicaTip™ 10 +/- 1 µm, New Objective, Woburn, MA, US) mounted on a Nanolockspray source of a SYNAPT™ G1 HDMS mass spectrometer (Waters). Accurate mass data were collected in a data independent positive mode of acquisition (MS^E) (selected *m/z* range 125 to 2000 Da). The $LCMS^E$ data were processed using the ProteinLynx Global SERVER™ v2.5 (PLGS, Waters Corporation). A database containing 4380 protein entries from the closely related *Stenotrophomonas maltophilia* K279a (downloaded from the Uniprot website, April 2014), together with a decoy database consisting of the randomized entries of all the proteins, was interrogated by PLGS. The precursor and fragment ion tolerance were determined automatically. The default protein identification criteria used included a maximal protein mass of 250,000 Da, a detection of minimal 3 fragment ions per peptide, minimal 7 fragment ions per protein and minimal 1 peptide per protein. Carbamidomethyl-C (fixed) and methionine oxidation (variable) were selected as modifications. Maximally one missed cleavages and a false positive rate of 4% was allowed.

4.2.6 β-lactamase assay

OMVs (isolated from 25 ml culture, dissolved in 1 ml PBS) were mixed with 50 µl of a 0.5 mg/ml nitrocefin solution (1 mg nitrocefin in 100 µl dimethylsulfoxide, diluted to 2 ml with PBS). The OD_{490nm} was measured at different time points. The hydrolysis of nitrocefin by β-lactamases generates an absorbance shift from yellow (intact nitrocefin, 380 nm) to red (hydrolyzed nitrocefin, 490 nm), which was monitored using a SmartSpec™ 3000 spectrophotometer (BioRad, Hercules, CA).

For the β-lactamase assay on crude cell lysate, cells from 0.5 ml PEN stimulated culture were collected by centrifugation at 4,000 x g for 15 min. The cell pellet was suspended in 500 µl PBS,

an equal volume of acid-washed glass beads (1-1.25 mm) was added, and cells were lysed by shaking vigorously for 5 minutes at 2500 rpm. The lysate was transferred to a new Eppendorf and the volume adjusted to 1 ml with PBS. Insoluble material was removed by centrifugation at 16,000 x g for 15 min. β-lactamase activity in the lysate was determined as described above.

To differentiate between the L1 metallo-β-lactamase and the L2 serine-β-lactamase, prior to the assay, 50 mM ethylenediaminetetraacetic acid (EDTA) was added and incubated for 30 min at 4°C.

4.2.7 Extraction of antibiotics

From a 25 µg/ml antibiotic suspension (start concentration) in 1 ml OMVs (isolated from 25 ml culture, dissolved in 1 ml PBS) or 1 ml crude cell lysate (as described in 2.6), 40 µl was collected at different time-points and mixed with 160 µl ACN. After shaking (5 minutes at 2500 rpm), the mixture was centrifuged at 16,000 x g for 15 minutes. The supernatant was collected and dried in a SpeedVac (SC110, Thermo Savant, Holbrook, NY). Finally, the pellet was resuspended in 15 µl 10 mM ammonium formate for RP-HPLC analysis.

4.2.8 RP-HPLC analysis of antibiotics

Antibiotic extracts were separated by RP-HPLC on an Ettan LC system (GE Healthcare), controlled by Unicorn® 5.11 software. Briefly, the sample (10 µl) was separated on a Zorbax Eclipse Plus C18 column (4.6x100 mm, 3.5 µm; Agilent) at a flow rate of 1 ml/min, with mobile phases 10 mM ammonium formate (solvent A) and ACN (solvent B) (ambient temperature). Analytes were separated with a 7.5 min gradient, going from 2% to 90% solvent B. Eluting compounds were detected with a UV detector (IPM: 298 nm, AMX: 230 nm, TIC: 220 nm).

4.2.9 Antibiotic tolerance plate assay

Cultures from *S. maltophilia*, *P. aeruginosa* and *B. cenocepacia* were grown in a 96-well plate, with different concentrations of the antibiotics IPM, AMX or TIC, with or without OMVs derived from a PEN stimulated *S. maltophilia* culture. Briefly, 50 µl of LB with a certain concentration of antibiotics (0, 1, 5, 10, 50, 100, 500, 1000, 5000 µg/ml) were mixed with 50 µl LB (control) or 50 µl OMVs in LB (OMVs from 50 ml culture in 1 ml LB) and to this mixture, 5 µl of cells were added (culture diluted to OD_{600nm} 0.2). Cultures were grown at 37°C for 16 h, after which the OD_{600nm} was measured with a 680XR micro plate reader (Bio-Rad, Hercules, CA).

4.2.10 Biofilm plate assay

Cultures from *S. maltophilia*, *P. aeruginosa* and *B. cenocepacia* were grown in a 96-well plate, with or without OMVs derived from a PEN stimulated *S. maltophilia* culture. Briefly, 50 µl of cells (culture diluted to OD_{600nm} 0.4) were mixed with 50 µl of LB (control) or OMVs in LB (OMVs from 25 ml culture in 500 µl LB). Cultures were grown at 37°C for 4 h to allow adhesion. In a third condition, the OMVs were first added to the wells and incubated for 1 h at 37°C. Then the supernatant was removed and the wells were washed with 300 µl of PBS (2x), after which 50 µl of cells and 50 µl of LB was added, followed by 4 h of adhesion. After adhesion, the culture supernatant (non-adhered cells) was removed, and the wells were washed with 300 µl PBS (2x). Finally, 100 µl of fresh LB medium was added to each well, and the plate was incubated at 37°C for 18 h.

Biofilm was quantified with crystal violet as described by Peeters et al. (2008) (two biological replicates, each with two technical replicates). Briefly, biofilm was fixed by adding 100 µl methanol to the cultures (15 min incubation), after which the wells were emptied and air dried. Then 100 µl of a crystal violet (0.1% w/v in water) was added to the wells. After 20 min incubation excess crystal violet was removed and the wells were rinsed with running tap water. Finally,

bound crystal violet was solubilized with 150 µl 33 % acetic acid and the absorbance was measured at 595 nm.

4.3 Results

4.3.1 Characterization of penicillin G induced OMVs

Previous work has shown that the exposure of *S. maltophilia* cells to the β-lactam antibiotic IPM led to a significant increase in the secretion of OMVs. Proteomics on isolated OMVs also revealed that these included large amounts of the two chromosomal encoded β-lactamases, the L1 metallo-β-lactamase and the L2 serine-β-lactamase (Chapter 3; Devos et al., 2015). In this study, OMVs were used from PEN stimulated cultures (1 mg/ml, sub-lethal concentration; Addendum A4). When OMVs were quantified with light scattering based single particle tracking, the amount of particles detected was comparable to the amount measured after IPM stimulation (2.91e12 particles/ml; Addendum A5). In addition, the OMVs were analyzed by fluorescent single particle tracking after incubation with the membrane specific fluorescent PKH67 label, which allows to quantify exclusively true membranous particles. Again, the results were similar as obtained for IPM (Addendum A5).

PEN-induced OMVs were also subjected to a 2D-LCMS proteomics study to identify the protein cargo. Both β-lactamases were again found to be included into OMVs. Furthermore, the OMV protein profile is strongly comparable to that of IPM-induced OMVs (87 % of identified protein were also identified in IPM OMVs; Addendum A6) (Chapter 3; Devos et al., 2015). The 2D-LCMS data was subjected to a label-free quantitative analysis with Progenesis™ (Nonlinear Dynamics, Newcastle, UK), but no big differences in protein abundances were observed (Addendum A6). Overall, these results indicate that OMVs obtained from PEN treated cells are very similar to those induced by IPM treatment and that their biogenesis, structure and composition is a result from the cell stress provoked by the inhibition of peptidoglycan synthesis.

4.3.2 OMV-associated β-lactamase activity

The β-lactamase activity of intact OMVs was examined (isolated from a 25 ml culture stimulated with 1 mg/ml PEN) using a nitrocefin β-lactamase assay (25 µg nitrocefin). The results show rapid nitrocefin hydrolysis by the β-lactamase packed OMVs (Figure 13, A). The rate of hydrolysis, derived from the linear part of the curve (5-15 min), was calculated as 0.571 µg/min (Addendum A7). In order to assess the contribution of the L1 metallo-β-lactamase, the same assay was performed after incubating the OMVs with the zinc-chelating agent EDTA (Hu et al., 2008). The initial rate of hydrolysis was now 0.262 µg/min, about half of the rate observed without EDTA. This demonstrates the OMV-associated activity of both β-lactamases. Remarkably, the β-lactamase assay on crude culture lysate obtained after PEN stimulation showed almost complete activity loss after the addition of EDTA (Figure 13, B). This points to a dominant role for L1 in cellular β-lactam resistance, as previously reported (Van Oudenhove et al., 2012). However, OMVs seem to have an equal activity distribution between L1 and L2. This could be explained by a more balanced targeting of both β-lactamases towards the OMVs, or the periplasm.

4.3.3 OMV-mediated degradation of β-lactam antibiotics

For the detection and quantification of hydrolysis of β-lactam antibiotics by PEN generated OMV samples, a fast and robust RP-HPLC method was set up. IPM, AMX and TIC were analysed with a short-gradient RPLC method (7.5 min gradient on a C18 Zorbax Eclipse Plus column) and each monitored with their optimal absorbance wavelength (IPM: 298 nm, AMX: 230 nm, TIC: 220 nm). The linear dynamic range was set from 50 ng to 5 µg (Addendum A8). For IPM, two peaks were detected, representing its two tautomeric forms. This was previously described, and was found to be stable (Verdier et al., 2011). Therefore, to perform the calculations of OMV-mediated degradation of IPM, only the first peak was considered.

To explore the potential of OMVs to degrade the different β-lactam antibiotics, OMVs (isolated from a 25 ml culture stimulated with 1 mg/ml PEN) were exposed to 25 µg/ml of IPM, AMX or

TIC. Reaction products were extracted immediately after starting the treatment (t0), and after 10 minutes (t10), 20 minutes (t20) and 30 minutes (t30) incubation at room temperature, and analyzed by RPLC. All three β-lactams were gradually degraded by the β-lactamase-packed OMVs, albeit with different efficiency (Figure 14). IPM and TIC are degraded rather slowly, as compared to AMX which is rapidly degraded. Former studies reported on an equal rate of hydrolysis of L1 towards AMX and IPM, but a 10-fold higher rate of hydrolysis by L2 towards AMX as compared to IPM (Paton et al., 1994; Walsh et al., 1997).

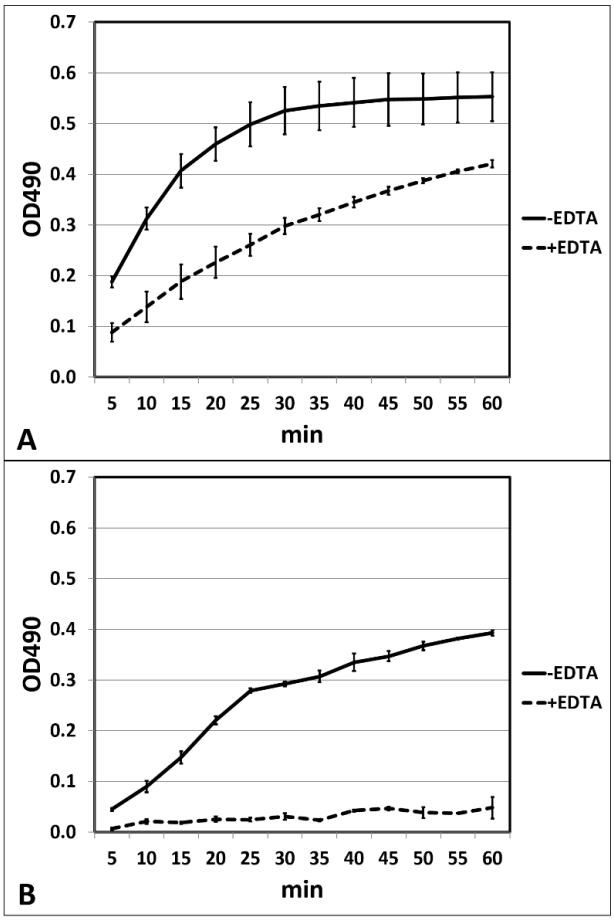

Figure 13. UV-VIS spectroscopy analysis of nitrocefin hydrolysis (OD490) at different time points during incubation with intact penicillin G induced OMVs (**A**), and crude penicillin G stimulated culture lysate (**B**), with and without the addition of EDTA. Error bars plot the standard deviation.

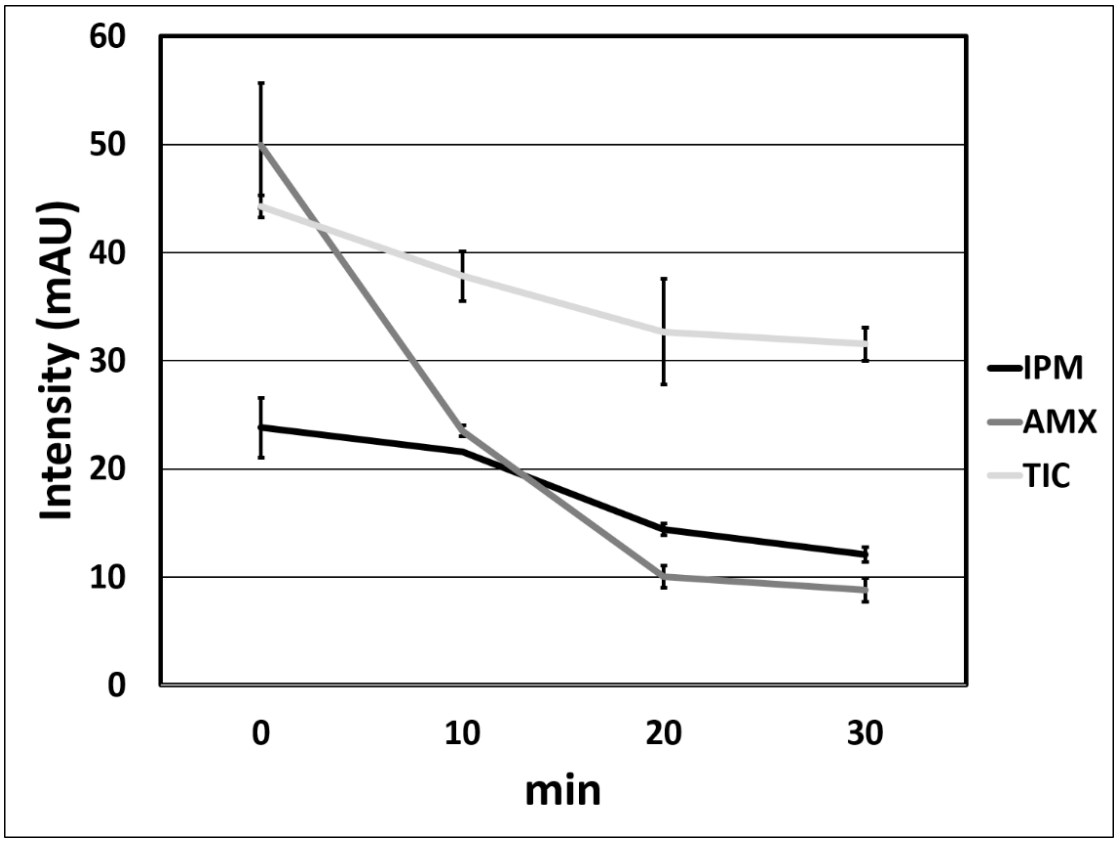

Figure 14. Results of the time-kinetic RP-HPLC analysis of imipenem (IPM), amoxicillin (AMX) and ticarcillin (TIC) at the start, and 10 min, 20 min and 30 min after incubation with intact penicillin G induced OMVs. Error bars plot the standard deviation.

4.3.4 Effect of OMVs on the antibiotic tolerance of *S. maltophilia*, *P. aeruginosa* and *B. cenocepacia*

As *S. maltophilia* is often part of polymicrobial communities, we investigated whether OMVs from *S. maltophilia* influence the tolerance of *P. aeruginosa*, *B. cenocepacia* and itself to different β-lactam antibiotics. *S. maltophilia*, *P. aeruginosa* and *B. cenocepacia* were grown in the presence of different concentrations of the β-lactam antibiotics IPM, AMX or TIC, with or without OMVs derived from a *S. maltophilia* culture exposed to PEN.

S. maltophilia shows a high resistance towards the three β-lactam antibiotics, with growth inhibition at 500 μg/ml, 2500 μg/ml and 250 μg/ml of IPM, AMX and TIC respectively (Figure 15, left column). When isolated OMVs (containing β-lactamases) were added, the inhibitory concentration increases to 2500 μg/ml for IPM and TIC, and even higher for AMX (> 2500 μg/ml). The effect of the antibiotics, and the OMVs, on *P. aeruginosa* and *B. cenocepacia* is very much alike (Figure 15, middle and right column). Both species are as resistant to AMX as *S. maltophilia*, and the addition of the OMVs also lead to an increase in the inhibitory concentration (> 2500 μg/ml). *P. aeruginosa* and *B. cenocepacia* are naturally less resistant to IPM and TIC, but the presence of *S. maltophilia* OMVs drastically increases the antibiotic tolerance for these species. A 100-fold increase in inhibitory concentration was observed: from 2.5 μg/ml to 250 μg/ml and from 25 μg/ml to 2500 μg/ml for IPM and TIC respectively.

4.3.5 Influence of OMVs on biofilm formation

We also analysed the influence of *S. maltophilia* derived OMVs on biofilm formation of *S. maltophilia* itself and of *P. aeruginosa* and *B. cenocepacia*. Cultures were incubated in the presence of OMVs ('OMV'), in the absence of OMVs ('control'), or in the absence of OMVs but after pre-incubation of the growth chamber with OMVs ('block'). The OMVs have a clear negative effect on biofilm formation in *S. maltophilia* itself, possible through blocking of the surface (Figure 16). Indeed, when the growth chamber was pre-incubated with OMVs, followed by a washing step and the addition of fresh culture (without OMVs), the same decrease was observed. In contrast, the OMVs of *S. maltophilia* have a stimulatory effect on biofilm formation in *P. aeruginosa* and *B. cenocepacia*.

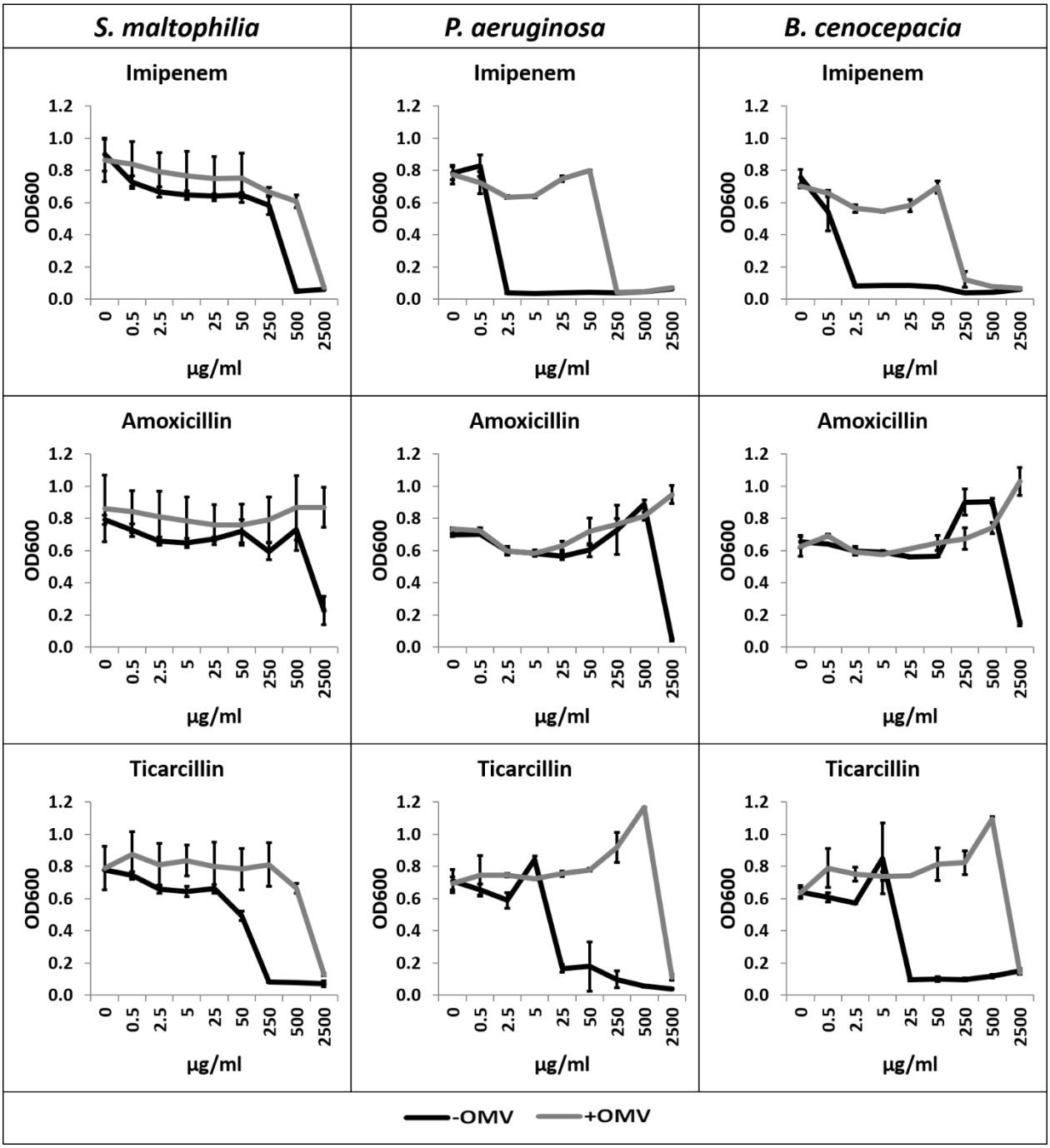

Figure 15. Optical density (OD600) of *S. maltophilia*, *P. aeruginosa* and *B. cenocepacia* cultures exposed to different concentrations of imipenem, amoxicillin and ticarcillin, in the absence and presence of penicillin G induced OMVs. Error bars plot the standard deviation.

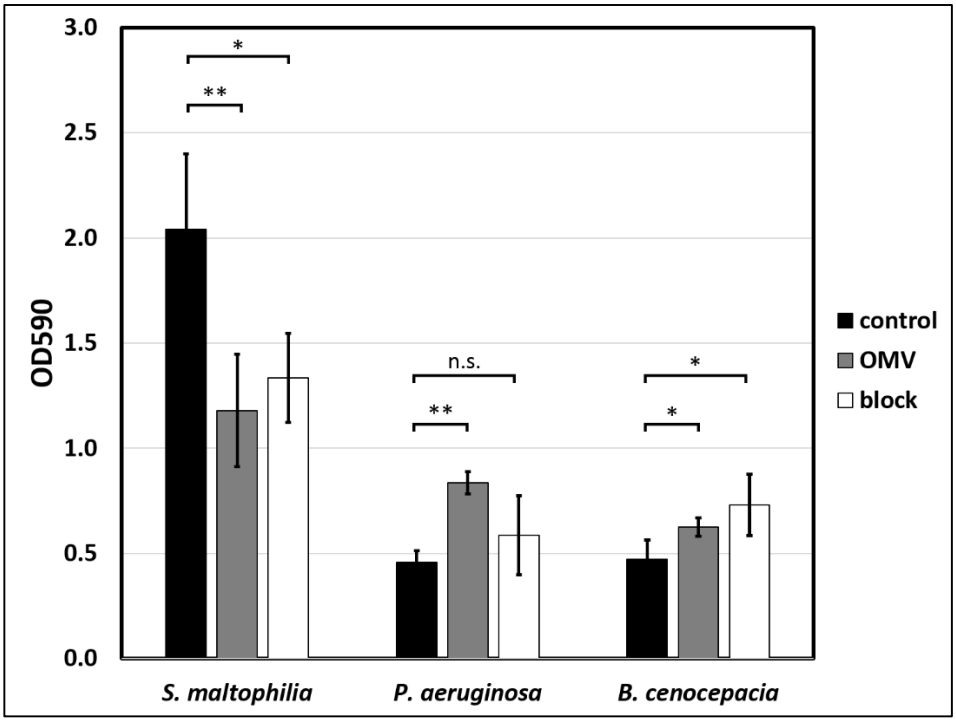

Figure 16. Crystal violet biofilm quantification analysis of *S. maltophilia*, *P. aeruginosa* and *B. cenocepacia* cultures grown without OMVs (control), together with penicillin G induced OMVs (OMV), and after pre-incubation of the growth chamber with penicillin G induced OMVs (block). Error bars plot the standard deviation. (Student's *t*-test; n = 4; *, $p < 0.05$; **, $p < 0.005$; n.s., not significant).

4.4 Discussion

Exposure of *S. maltophilia* cells to β-lactam antibiotics such as imipenem (Chapter 3; Devos et al., 2015) and penicillin G (present study) leads to a significant increase in the biogenesis of OMVs, which are packed with β-lactamases. Since the *S. maltophilia* L1 and L2 β-lactamases are targeted to the periplasm, this raises the question whether their presence in OMVs is merely accidental considering their location, or if the β-lactamases are specifically targeted for OMV-associated secretion.

We showed here by means of a nitrocefin assay that β-lactamase-packed OMVs (generated by stimulating cells with penicillin G) indeed exhibit β-lactamase activity. OMVs provide the enzymes shelter against proteases, keeping them stable and active for longer periods (Aldick et al., 2009). The extracellular β-lactamase activity associated with OMVs could hereby also affect other species, possibly co-habitants in a polymicrobial community. It was already shown that *S. maltophilia* often lives together with the species *P. aeruginosa* and *B. cenocepacia*, especially in lungs of cystic fibrosis patients where these are found in polymicrobial biofilm communities (Raidt et al., 2015). Here we show that the exposure of *S. maltophilia* to β-lactam antibiotics leads to the secretion of β-lactamase-packed OMVs, which are in turn capable of protecting not only other *S. maltophilia* cells, but also *P. aeruginosa* and *B. cenocepacia*, against β-lactam antibiotics. Although it is not clear whether *S. maltophilia* can be considered as a true CF pathogen (Hansen, 2012), its ability to secrete OMVs upon antibiotic stress can influence the susceptibility of the pathogens to antibiotic treatment.

For many organisms, OMVs represent a common constituent of biofilm, and studies have also shown a role in cell aggregation and biofilm initiation (Schooling and Beveridge, 2006). Here we observed a negative effect of penicillin G induced *S. maltophilia* OMVs on *S. maltophilia* biofilm formation. Most likely the OMVs attach to available surfaces and hereby prevent cell attachment and subsequent biofilm formation. This was confirmed by first incubating the growth chamber with OMVs, after which the chamber was washed and culture was added without OMVs. The

same negative effect is observed in the closely related plant pathogen *Xylella fastidiosa*. OMVs of *X. fastidiosa* block surfaces, hereby inhibiting biofilm formation, leading to an increased virulence through deeper plant colonization (Ionescu et al., 2014).

On the other hand, *S. maltophilia* OMVs stimulated biofilm formation of *P. aeruginosa* and *B. cenocepacia*, regardless if OMVs were mixed with the culture or if the growth chamber was pre-incubated with the OMVs. This suggests that *S. maltophilia* OMVs might accommodate cell-cell interactions and/or communication that promote cell attachment and aggregation. It was already proven that diffusible signal factors (DSF) produced by *S. maltophilia* can influence *P. aeruginosa* and *B. cenocepacia* behavior (Ryan and Dow, 2011; Twomey et al., 2012). Signaling molecules packed in OMVs could potentially be delivered to other cells through vesicle docking and uptake by these cells. Our work indicates that OMV biology is complex and that inter-species interactions should be considered when developing OMVs as an antimicrobial drug target.

4.5 References

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- Chapter 5 -

The effect of ciprofloxacin on membrane vesicle secretion in *Stenotrophomonas maltophilia*

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Author contributions

I prepared the OMVs and performed the 2D-LCMS^{E} OMV profiling experiment and the OMV toxicity experiments. JV assisted in OMV preparation and OMV proteome analysis. All microscopy was carried out by WVP, with the assistance of Wim Van Den Broeck for TEM, and Misha Kudryashev and Henning Stahlberg for cryo-TEM. SS carried out the OMV quantification experiments, with help of JV, and supervision of KR. GV provided technical support for all measurements with the NanoAcquity UPLC® system and the SYNAPT^{TM} G1 HDMS mass spectrometer. I wrote the manuscript. BD supervised the research and edited the manuscript.

Abstract

Alike β-lactam antibiotics, the exposure of *Stenotrophomonas maltophilia* to the fluoroquinolone ciprofloxacin leads to stress-induced membrane vesicle secretion. Ciprofloxacin-induced vesicles differ considerable from previously characterized imipenem-induced outer membrane vesicles (OMV). Ciprofloxacin leads to the secretion of two distinct populations of vesicles: small OMVs morphologically similar to vesicles produced upon imipenem challenge, and larger vesicles containing both inner- and outer membrane (outer-inner membrane vesicles, OIMVs) with filamentous structures at the surface. The ciprofloxacin-induced vesicles have a strong toxic effect towards *Pseudomonas aeruginosa*, presumably caused by the phage tail bactericidal protein maltocin P28, carried by the vesicles.

Keywords

Ciprofloxacin, outer-inner membrane vesicles, SOS response, phage tail, maltocin

5.1 Introduction

The release of bacterial outer membrane vesicles (OMVs) is widely described as a cellular response to stress conditions, and is now recognized as a true secretion system (Schwechheimer and Kuehn, 2015). The spheroid membrane nanoparticles (20-300 nm) are formed through outer membrane budding, whilst encapsulating cell material, followed by pinching off into the environment (Mashburn-Warren et al., 2008; Bonnington and Kuehn, 2014). OMV secretion is conserved in Gram-negative bacteria, both pathogenic as non-pathogenic (Kuehn and Kesty, 2005), and the vesicles have important biological functions related to virulence, biofilm formation and antibiotic resistance (Olsen and Amano, 2015; Chapter 4).

Former studies on multidrug resistant *Stenotrophomonas maltophilia* demonstrated a significant increase in OMV secretion after exposure to β-lactam antibiotics (Devos et al., 2015). The two chromosomal encoded β-lactamases were proven to be an important constituent of such OMVs, and they are able to establish an extracellular OMV-mediated degradation of β-lactam antibiotics (Chapter 4). The effect of β-lactam antibiotics on bacterial cell vesiculation can be in part explained by their mechanism of action. The accumulation of peptidoglycan degradation products in the periplasmic space and the loss of peptidoglycan-outer membrane crosslinks are proposed as possible causes of OMV biogenesis (Haurat et al., 2014; Schwechheimer and Kuehn, 2015). However, how other stimuli are able to induce OMV biogenesis is still not clear. For example, high concentrations (1 mM) of the *S. maltophilia* diffusible signal factor cis-11-methyl-2-dodecenoic acid (DSF) also led to substantial OMV secretion, and to a lesser extent the *Burkholderia cenocepacia* cis-2-dodecenoic acid (BDSF) did too (Devos et al., 2015). In contrast, deletion of the *rpfF* gene in the related plant pathogen *Xylella fastidiosa*, coding for the DSF synthase rpfF, stimulated the OMV biogenesis process (Ionescu et al., 2014). This points to a possible important role of the DSF quorum sensing system in regulating OMV biogenesis.

Considering that cell vesiculation is often a mechanism to cope with cell stress (Schwechheimer and Kuehn, 2015), also other types of antibiotics can lead to increased membrane vesicle

secretion. In *Pseudomonas aeruginosa*, the fluoroquinolone ciprofloxacin induces OMV secretion which was proven to be linked to the SOS response provoked by DNA damage through DNA gyrase inhibition (Maredia et al., 2012). The ciprofloxacin-induced vesiculation was also observed in *Acholeplasma laidlawii*, where it was demonstrated that the vesicles mediate ciprofloxacin export (Medvedeva et al., 2014). These findings all suggest that bacterial vesiculation is a regulated process that is an important aspect of the antibiotic stress response and in antibiotic resistance.

In the present work, we studied the effect of the fluoroquinolone ciprofloxacin on *S. maltophilia* membrane vesicle secretion. We compared the protein content of ciprofloxacin raised vesicles with OMVs resulting from β-lactam antibiotic cell wall stress, and analyzed the effect of these vesicles on the viability of species with which *S. maltophilia* co-inhabits the lungs of cystic fibrosis (CF) patients. Ciprofloxacin is often prescribed to patients with CF, especially for treating chronic respiratory infections with *P. aeruginosa* (Langton Hewer and Smyth, 2014).

5.2 Material and methods

5.2.1 Materials

ULC-MS grade water and acetonitrile (ACN) was procured from Biosolve (Valkenswaard, The Netherlands). Sequencing grade modified trypsin was obtained from Promega (Madison, WI, US). Acetic acid was purchased from VWR. All other chemicals and antibiotics were purchased from Sigma-Aldrich (St. Louis, MO, US).

5.2.2 Bacterial cell culture

The bacterial strains *Stenotrophomonas maltophilia* 44/98 (LMG 26824), *Pseudomonas aeruginosa* PAO1 (LMG 24986), and *Burkholderia cenocepacia* type strain (LMG 16656) were grown aerobically overnight in Luria Broth (LB) until the stationary phase. The cell suspensions were then diluted to an OD_{600nm} of 0.2 in fresh medium, and allowed to grow further under the same conditions.

5.2.3 Preparation of outer membrane vesicles

S. maltophilia (50 ml cultures) were grown for 2 hours until the mid-exponential growth phase (OD_{600nm} = 0.65-0.75), and then grown for an additional 3 hours in the presence of antibiotics (25 µg/ml imipenem or 2 µg/ml ciprofloxacin). Cells were pelleted by centrifugation at 4,000 x g for 15 min. The culture supernatant was filtered through a 0.2 µm V25 vacuum filter (Sarstedt, Numbrecht, GE), and the OMVs were pelleted by ultracentrifugation at 100,000 x g for 1 h (Avanti J-30I, Beckman Coulter, Fullerton, CA). One milliliter of the filtered culture supernatant was spread onto an LB agar plate and incubated at 37°C for 24 h to confirm the absence of intact, living cells.

5.2.4 OMV quantification with single particle tracking

OMVs from 50 ml cultures were harvested as described above, and the pellet was dissolved in 100 µl phosphate buffered saline (PBS). The OMV concentration and size was determined by light scattering based single particle tracking using a NanoSight LM10-HS instrument (NanoSight, Amesbury, UK; Dr. A. Hendrix, Laboratory of Experimental Cancer Research, Ghent University Hospital) equipped with a 405 nm laser. Prior to analysis, the purified OMVs were diluted in PBS-buffer (Invitrogen). Movies of 60 seconds were recorded and analyzed with the NTA Analytical Software version 2.3. Each individual sample was diluted and measured 3 times. Calculations were performed according to Van der Pol et al. (2010).

5.2.5 Transmission electron microscopy

A 4 µL drop of isolated OMVs was placed on a Formvar/carbon-coated cupper grid, made hydrophilic by glow discharging for 30 s. The grid was then washed by placing it sequentially onto 5 drops of milliQ water. After these washing steps, the grid was placed on 2 drops of 2% uranyl acetate and incubated for 30 to 40 s. The grid was blotted with filter paper between each washing step. The created specimens were examined using a JEM 1400 Plus transmission electron microscope (JEOL, Tokyo, Japan) operating at 80 kV equipped with a 11 MegaPixel Bottom-Mounted Quemesa CCD camera (Olympus, Germany).

For cryo-TEM, the OMVs were supplemented with 5% of 10 nm gold beads, placed on holey carbon grids (Quantifoil Micro Tools GMBH, Germany), quickly vitrified using a FEI Vitrobot IV (FEI Corp, Hillsboro), and imaged at liquid nitrogen temperatures in an FEI Titan Krios (FEI Corp, Hillsboro) operated at 300 kV acceleration voltage and equipped with a GIF and a post-GIF K2 DED detector (Gatan Inc, Pleasanton). Cryo-images were obtained using a magnifiction of 33kX and a defocus of 5µm.

5.2.6 OMV protein extraction and digestion

OMV proteins were extracted by dissolving the OMV pellet in 1 ml 8 M urea in 50 mM Tris-HCl pH 8. Proteins were precipitated with trichloroacetic acid (20%) and consequently the pellet was washed twice with ice-cold acetone, and finally dissolved in 2 M urea in 50 mM ammonium bicarbonate (pH 8). The protein concentration was assessed using the Coomassie Plus Bradford™ Assay kit (Thermo Scientific, San Jose, CA, US). Proteins were reduced with 10 mM dithiothreitol for 30 min at 60 °C, alkylated with 20 mM iodoacetamide at ambient temperature for 30 min, and then digested with trypsin (1:50 w/w) overnight at 37 °C.

5.2.7 2D-LCMS^E analysis

Peptide mixtures (0.5 µg/µl in 100 mM ammonium formate, pH 10) were separated on a NanoAcquity UPLC® system (Waters Corporation, Milford, MA) in 2D mode. For the first dimension (high pH), solvent A1 and B1 were composed of 20 mM ammonium formate in water and ACN (pH 10), respectively. For the second dimension (low pH), solvent A2 and B2 were composed of 0.1% formic acid in water and 0.1% formic acid in ACN, respectively. The sample (0.5 µg) was loaded onto an Xbridge® Peptide BEH C18 NanoEase™ column (130 Å, 5 µm, 300 µm x 50 mm; Waters) at 3% solvent B1 at 2 µL/min. Peptides were eluted from the first dimension column in 6 fractions (11.1%, 14.5%, 17.4%, 20.8%, 45% and 65.0% of solvent B1), and fractions were trapped on a Symmetry® C18 trapping column (5 µm, 180 µm x 20 mm; Waters). Each fraction was separated on a Acquity UPLC® M-Class HSS T3 column (1,8 µm, 75 µm x 250 mm; Waters) at 40°C at 280 nL/min by increasing the acetonitrile concentration from 5-50% B2 over 60 min.

The outlet of the column was directly connected to a PicoTip Emitter (uncoated SilicaTip™ 10 +/- 1 µm, New Objective, Woburn, MA, US) mounted on a Nanolockspray source of a SYNAPT™ G1 HDMS mass spectrometer (Waters). The time-of-flight (TOF) analyzer was externally calibrated with MS/MS fragments of human [glu^1]-fibrinopeptide B (Glu-fib) from *m/z* 72 to 1285, and the data was corrected post-acquisition using the monoisotopic mass of the doubly charged precursor

of Glu-fib (*m/z* 785.8426) (lock mass correction). Accurate mass data were collected in a data independent positive mode of acquisition (MS^E) by alternating between low (5 V) and high (ramping from 15 to 35 V) energy scan functions (Geromanos et al., 2009). The selected *m/z* range was 125 to 2000 Da. The capillary voltage was set to 3.0 kV, the sampling cone voltage was 26 V and the extraction cone voltage on 4 V. The source temperature was set on 80 °C.

The $LCMS^E$ data were processed using the ProteinLynx Global SERVER™ v2.5 (PLGS, Waters Corporation). A database containing 4380 protein entries from the closely related *Stenotrophomonas maltophilia* K279a (downloaded from the Uniprot website, April 2014), together with a decoy database consisting of the randomized entries of all the proteins, was interrogated by PLGS. The precursor and fragment ion tolerance were determined automatically. The default protein identification criteria used included a maximal protein mass of 250,000 Da, a detection of minimal 5 fragment ions per peptide, minimal 10 fragment ions per protein and minimal 2 peptides per protein. Carbamidomethyl-C (fixed) and methionine oxidation (variable) were selected as modifications. Maximally one missed cleavages and a false positive rate of 4% was allowed.

5.2.8 OMV toxicity assay

Cultures from *S. maltophilia*, *P. aeruginosa* and *B. cenocepacia* were grown in a 96-well plate, with or without OMVs derived from imipenem or ciprofloxacin stimulated *S. maltophilia* cultures. Briefly, 100 µl LB (control) or 100 µl OMVs in LB (OMVs from 2.5 ml culture) were mixed with 5 µl of cells (from culture diluted to OD_{600nm} 0.2). Cultures were grown at 37°C for 16 h, after which the OD_{600nm} was measured with a 680XR micro plate reader (Bio-Rad, Hercules, CA). A total of 4 replicate analysis were performed: 2 biological OMV replicates mixed with 2 biological culture replicates.

To address the importance of the vesicles and vesicle associated proteins in toxicity, the assay was repeated on *P. aeruginosa* cultures, but after treatment with 1 mg/ml proteinase K, 2.5 %

Triton X-100, or both. The vesicles were incubated for 30 minutes at room temperature before mixing with the cells.

5.3 Results

5.3.1 OMV quantification and size determination

The previously reported increase in vesiculation in *S. maltophilia* is supposed to be a direct consequence of the cell wall damage inflicted by the β-lactam type antibiotics that were used (i.e. accumulation of peptidoglycan degradation products, loss of peptidoglycan crosslinking and/or peptidoglycan-outer membrane connections). We compared the effect of the β-lactam antibiotic imipenem and the fluoroquinolone antibiotic ciprofloxacin by quantifying and sizing the vesicles with light scattering based single particle tracking. As reported before, imipenem led to a substantial increase in vesiculation (Figure 17). However, while not supposed to have a direct effect on peptidoglycan stability, ciprofloxacin also led to a significant increase in vesicle secretion, which makes antibiotic stress a potential inducer of cell vesiculation, in a broader sense. Although the vesicle concentration is lower in ciprofloxacin-stimulated cultures, the average size of the vesicles is larger (imipenem: 140 nm, ciprofloxacin: 231 nm; Addendum A9)

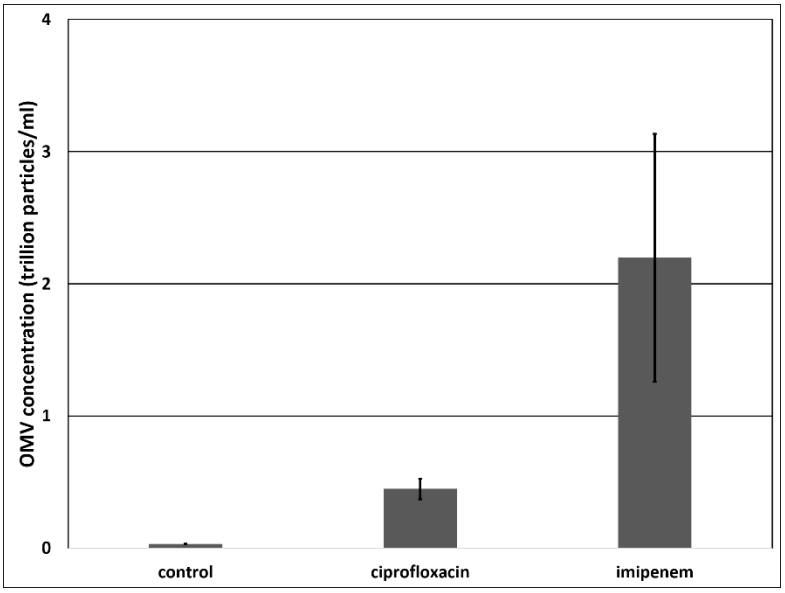

Figure 17. Quantification of membrane vesicles isolated from unstimulated, and imipenem and ciprofloxacin stimulated *S. maltophilia* cultures. Vesicles were quantified with light scattering based single particle tracking. Error bars plot the standard deviation.

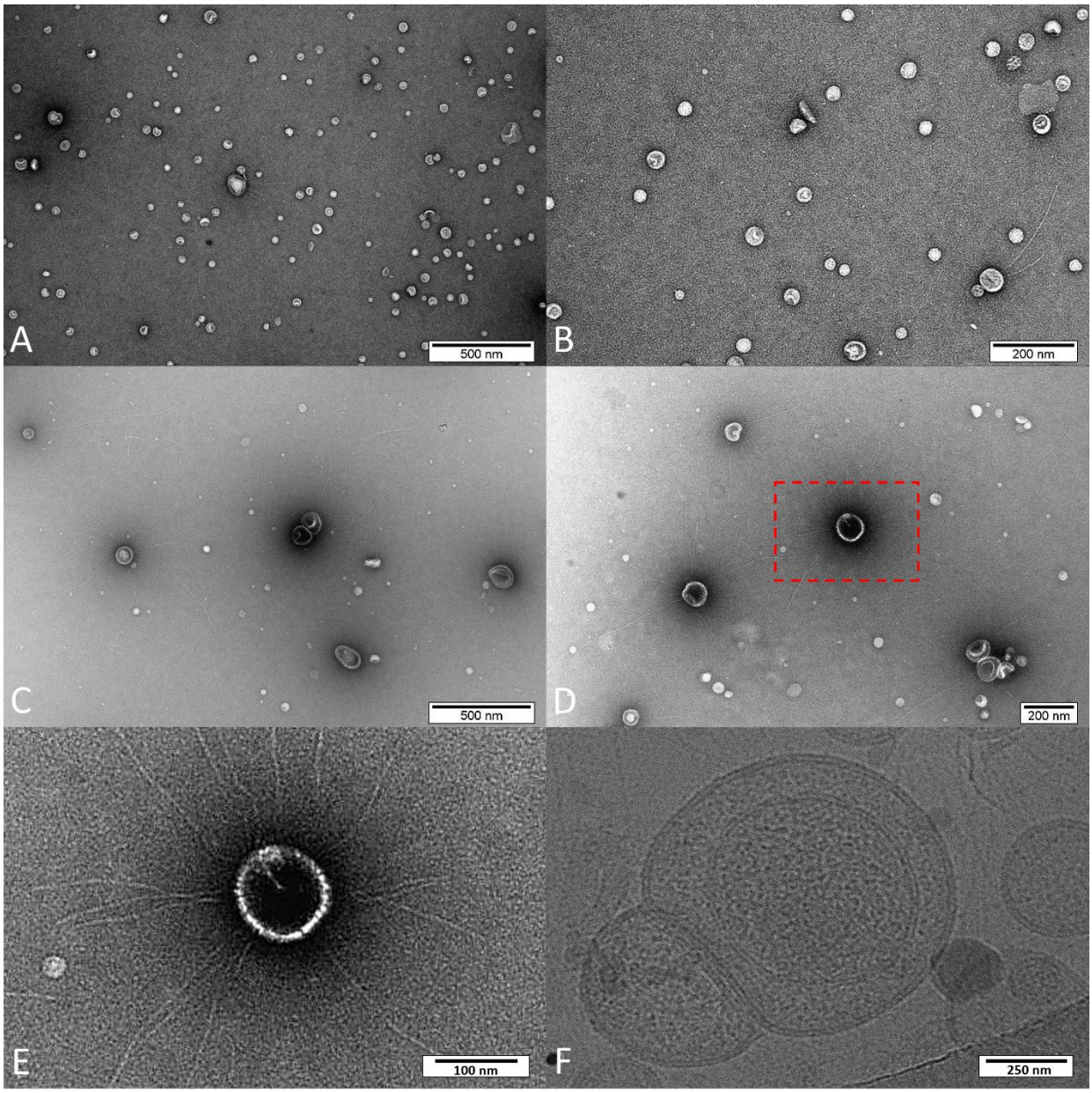

Figure 18. Transmission electron microscopy visualization of isolated membrane vesicles from imipenem (**A-B**) and ciprofloxacin (**C-D**) stimulated *S. maltophilia* cultures. Filament structures on ciprofloxacin-induced vesicles (**E**; picture zoom of red box in D). Cryo-TEM image of ciprofloxacin-induced outer-inner membrane vesicle (**F**).

5.3.2 Vesicle morphology

The vesicles isolated from imipenem- and ciprofloxacin-stimulated cultures were visualized with transmission electron microscopy (TEM) (Figure 18). Confirming data from the single particle tracking analysis, the ciprofloxacin-stimulated cultures produced overall less vesicles, but bigger vesicular structures were observed. Imipenem stimulation leads to vesicles with an estimated size of around 50 nm (Figure 18, A-B), while ciprofloxacin stimulation also leads to vesicles with an estimated size between 100-200 nm, next to the smaller vesicles as observed with imipenem (Figure 18, C-D) (the smaller size in TEM is potentially a consequence of vesicle shrinking during fixation). Moreover, these bigger vesicles often carry fiber-like structures at the surface (Figure 18, E). Further analysis with cryo-TEM revealed the presence of both inner and outer membrane in most of the larger vesicles (Figure 18, F). Therefore, the large vesicles are actually outer-inner membrane vesicles (OIMV), a different type of secreted membrane vesicles (Perez-Cruz, 2015).

5.3.3 Proteomic analysis of imipenem- and ciprofloxacin-induced OMVs

OMVs from imipenem- and ciprofloxacin-stimulated cultures were isolated, and extracted proteins were digested and analyzed with 2D-LCMS^E. Proteins were only further considered when identified in all three biological replicate samples. A total of 184 proteins were identified in the imipenem-induced OMV samples, and 170 proteins were identified in the ciprofloxacin-induced OMV samples (Addendum A10). Proteins were further annotated with Psortb v3.0 and UniProt (gene ontology).

The protein profile from imipenem-induced OMVs is very much alike as previously obtained (more than 90% of the proteins were identified in the former proteomics experiment) (Devos et al., 2015). However when comparing the protein profiles of both types of OMVs, the large amount of cytoplasmic proteins identified in the ciprofloxacin samples is striking: 36.5% of the proteins are predicted to be cytoplasmic as compared to 14.1% in the imipenem samples (Figure 19). Presumably this is due to the presence of OIMVs in ciprofloxacin-stimulated cultures. Roughly half

of these are actually ribosomal proteins. Interestingly, among the ciprofloxacin-specific cytoplasmic proteins are the Recombinase A protein (RecA) involved in DNA repair and inducing the SOS response to DNA damage, and the ciprofloxacin target itself, DNA gyrase. Several other cytoplasmic proteins are also involved in the cellular stress response, for example the lon protease protein, the universal stress protein, the general stress protein CTC, and the poly-hydroxy-butyrate synthesis protein. Other (often cytoplasmic) proteins are involved in protein folding, for example the GroEL-GroES chaperonin complex, chaperone proteins DnaK (heat shock protein 70, HSP70) and HtpG (high temperature protein G), and several peptidyl-prolyl isomerases including the periplasmic SurA chaperone were found. However, this last group is found in both ciprofloxacin- and imipenem-induced membrane vesicles. In addition, several superoxide dismutases (SodA, SodC1, SodC2) and a subunit from the alkyl hydroperoxide reductase (AhpC) were identified in imipenem- and ciprofloxacin-induced vesicles, indicating oxidative stress in both conditions.

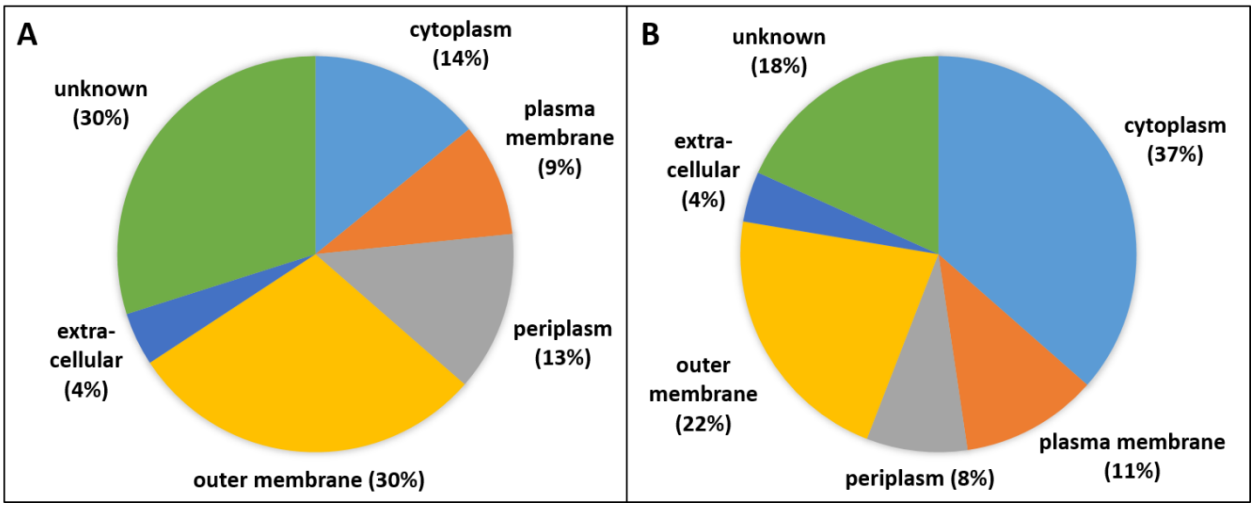

Figure 19. Protein location distribution of identified proteins from 2D-LCMS^E analysis of imipenem- (**A**) and ciprofloxacin- (**B**) induced membrane vesicles. Protein location was annotated with Psortb v3.0 and UniProt (GO).

Interestingly, the ciprofloxacin samples contained more plasma membrane proteins, but less periplasmic and outer membrane proteins were identified. For example, several ATP synthase complex subunits (F-ATPase α-subunit, β-subunit, ε-subunit, γ-subunit, b-subunit), NADH-quinone oxidoreductase complex subunits (subunits B, C and D), and the succinate

dehydrogenase flavoprotein subunit were found, all involved in electron transport and oxidative phosphorylation. The ciprofloxacin samples also contained several components of the pyruvate dehydrogenase complex and proteins part of the citric acid cycle (2-oxoglutarate dehydrogenase complex, succinate dehydrogenase and malate dehydrogenase).

Finally, a cluster of phage-related proteins are exclusively present in ciprofloxacin-induced membrane vesicles: phage tail protein, major tail tube protein, major tail sheath protein, baseplate assembly protein, and phage-related protein. The identified major tail sheath protein was previously described in *S. maltophilia* strain P28 as maltocin, a phage tail-like bacteriocin (Liu et al., 2013).

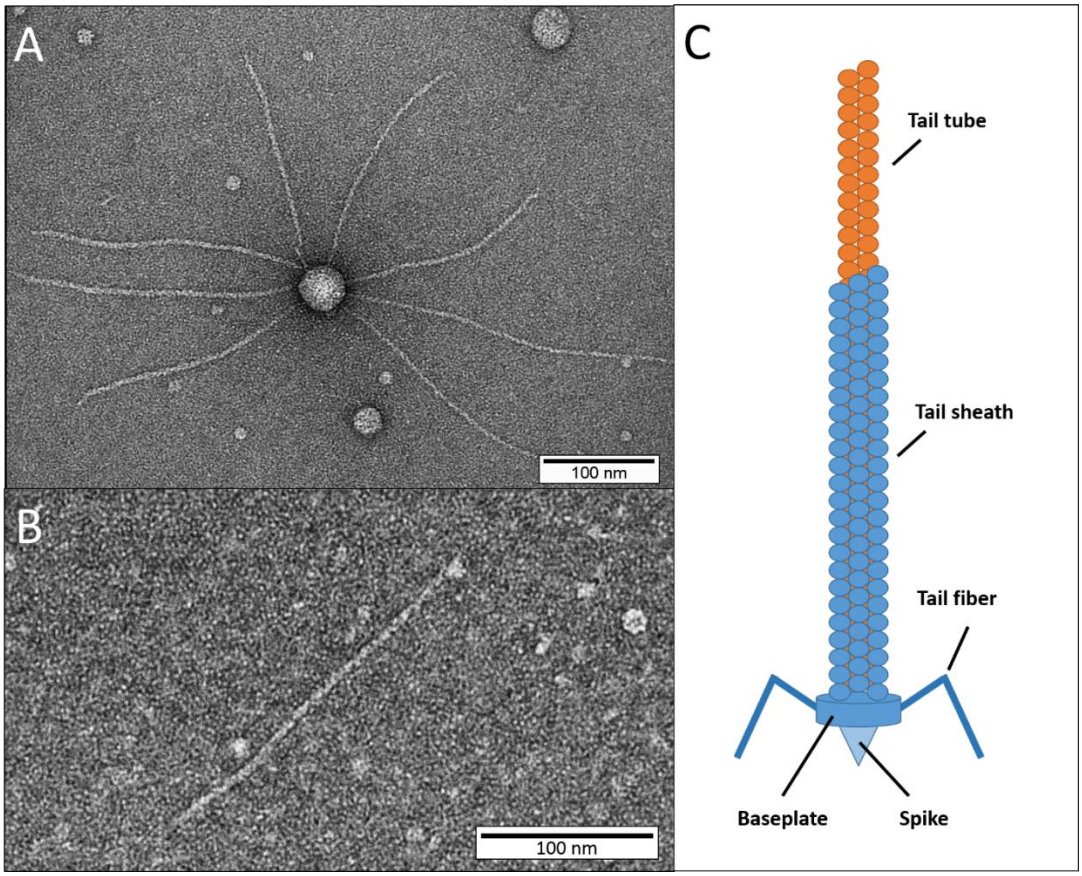

Figure 20. Transmission electron microscopy image of ciprofloxacin-induced vesicle carrying the maltocin tailocins (**A**). Detached maltocin after Triton X-100 treatment (**B**), morphologically comparable to the tailocin structure (**C**; figure adapted from Ghequire and De Mot, 2015).

5.3.4 Bactericidal effect of ciprofloxacin-induced vesicles

Maltocin is morphologically similar to the bacteriocin pyocin produced by *P. aeruginosa*. Maltocin and pyocin are phage-related bactericidal tailocins, multiprotein particles similar to phage tails (Ghequire et al., 2015). Microscopy (and proteomics) revealed the vesicle-mediated secretion by *S. maltophilia* of these phage tails after ciprofloxacin exposure (Figure 20, A). Vesicle-detached phage tails can be obtained by lysing the purified vesicles with Triton X-100 (2.5 %) (Figure 20, B). The observed structure corresponds well to the bacterial tailocins (Figure 20, C). Also, the tailocins showed strong resistance to protease treatment. There was no visible reduction in the amount of vesicle-associated tailocins after treating the vesicles with 1 mg/ml protease K (30 minutes at room temperature; data not shown).

The phage tail-like maltocin P28 has been shown to provide bactericidal activity against other strains of S. maltophilia then the P28 strain from which it was originally isolated (Liu et al., 2013). Therefore, we also addressed the bactericidal activity of the ciprofloxacin-induced membrane vesicles. Ciprofloxacin-induced vesicles, as well as imipenem-induced vesicles, were administered to cultures of *S. maltophilia* 44/98 itself, but also of *P. aeruginosa* PAO1 and *B. cenocepacia* type strain. Imipenem-induced vesicles did not lead to growth inhibition in any of the above cultures. Surprisingly, the ciprofloxacin-induced vesicles did had a very dramatic effect on the viability of *P. aeruginos*a PAO1 cultures, but not for the *S. maltophilia* and *B. cenocepacia* strains (Figure 21, A). *P. aeruginosa* PAO1 has a low MIC for ciprofloxacin (± 0.05 µg/µl; data not shown), so the toxic effect could be from residual ciprofloxacin in the membrane vesicle preparations. However, a negative control preparation with ciprofloxacin-medium did not lead to any growth inhibition towards *P. aeruginosa* PAO1 (data not shown).

To verify whether the toxicity towards *P. aeruginosa* PAO1 is dependent on the membrane vesicles, or on other vesicle-associated proteins, the bactericidal activity assay was repeated after treatment of the ciprofloxacin-induced vesicles with proteinase K (1 mg/ml), with Triton X-100

(2.5 %), or both. Apparently, the toxicity endures regardless of the used treatments (Figure 21, B).

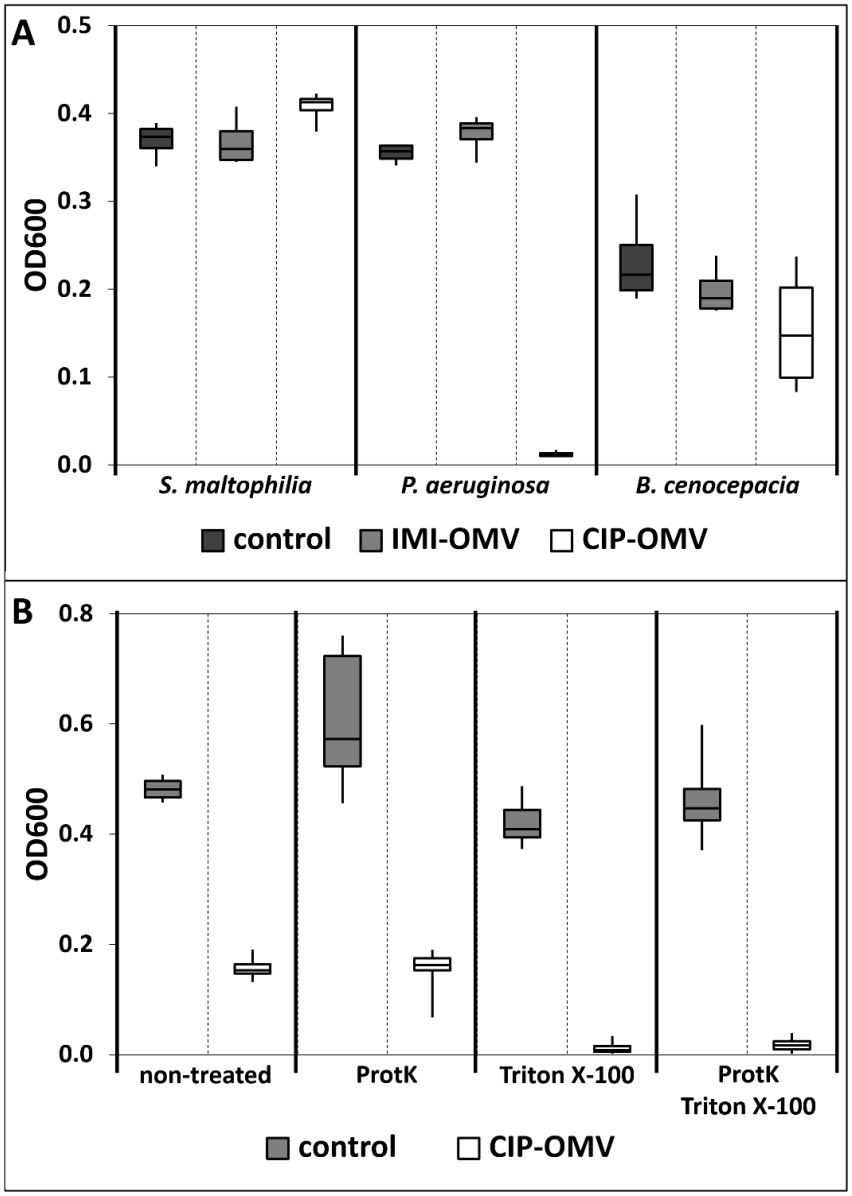

Figure 21. Membrane vesicle toxicity assay. (**A**) Boxplot showing optical density (OD600) of *S. maltophilia* 44/98, *P. aeruginosa* PAO1 and *B. cenocepacia* type strain cultures exposed to imipenem- (IMI-OMV) and ciprofloxacin-induced (CIP-OMV) membrane vesicles. (**B**) Boxplot showing OD600 of *P. aeruginosa* PAO1 cultures exposed to CIP-OMV, treated with proteinase K (1 mg/ml), Triton X-100 (2.5 %), and both.

5.4 Discussion

The release of membrane vesicles by Gram-negative bacteria is now recognized as a genuine secretion process, rather than a passive process, in response to different stimuli (Schwechheimer and Kuehn, 2015). In several studies it was shown that OMVs play important roles in β-lactam resistance (Ciofu et al., 2000; Schaar et al., 2011; Lee et al., 2013; Stentz et al., 2015). However, since vesiculation is often a strategy for cells to deal with stress, it should not be surprising that they are also involved in responses to other types of antibiotics.

The number of studies on OMV secretion in response to non-β-lactam antibiotics is scarce. However, few studies report on vesiculation after ciprofloxacin exposure. In *P. aeruginosa* it was shown that membrane vesicles are secreted in response to ciprofloxacin, and that the process is intimately linked to the cell SOS response caused by the fluoroquinolone-induced DNA damage (Maredia et al., 2012). Here we confirmed that ciprofloxacin also leads to an increase in vesicle secretion in *S. maltophilia* as revealed by light-scattering based single particle tracking. Electron microscopy showed a notable presence of two different sub-populations of vesicles after ciprofloxacin stimulation. Next to the 'classic' OMVs, i.e. comparable to those observed after imipenem stimulation, also many bigger vesicles were observed. Moreover, these bigger vesicles were often decorated at the surface with fibrous structures.

The ciprofloxacin-induced vesicles were subjected to a 2D-LCMS proteomics experiment, and the acquired protein profile was compared to that of imipenem-induced vesicles. Several proteins were identified that are involved in cell stress, oxidative stress and stress response to DNA damage (SOS), similar as reported in *P. aeruginosa* (Maredia et al., 2012). Also, the ciprofloxacin vesicles contained considerable more cytoplasmic proteins, and few more inner membrane proteins. Remarkably, many of those inner membrane proteins belong to protein complexes involved in primary metabolism, for example the citric acid cycle, electron transport chain and the ATP synthase complex for oxidative phosphorylation. This suggested that the larger vesicles in the samples are in fact outer inner membrane vesicles (OIMVs), recently reported as a different

type of bacterial membrane vesicle (Perez-Cruz et al., 2015). Indeed, cryo-TEM confirmed the presence of a double membrane layer in those bigger vesicles observed after ciprofloxacin stimulation.

The ciprofloxacin-induced vesicles were shown to contain a cluster of phage-related proteins, of which the identified major tail sheath protein was previously described in *S. maltophilia* strain P28 as maltocin, a phage tail bacteriocin, or tailocin (Liu et al., 2013). Maltocin is morphologically comparable with R-type pyocin tailocins produced by *P. aeruginosa* (Ghequire et al., 2015). The tailocin structure consist of a rigid tube inside a contractile sheath, and lipopolysaccharide-recognizing tail fibers attached to a base plate (Ghequire and De Mot, 2015). The tailocins are able to dock specific target strain cells and inject toxins. Their production was also shown to be associated with membrane vesicle formation (Toyofuku et al., 2014). Considering the bactericidal activity of maltocin, the ciprofloxacin-induced maltocin carrying vesicles were tested for toxicity towards *S. maltophilia* (strain 44/98), and the frequently cohabitating species *P. aeruginosa* (strain PAO1) and *B. cenocepacia* (type strain). Interestingly, the toxic effect of the vesicles is limited towards *P. aeruginosa*. It was previously demonstrated that imipenem-induced OMVs from *S. maltophilia* 44/98 are packed with β-lactamases and increased the β-lactam tolerance, and hereby the viability, of *P. aeruginosa* PAO1 during β-lactam stress (Chapter 4). In contrast, the exposure of *S. maltophilia* 44/98 to ciprofloxacin leads to the secretion of vesicles that are toxic for *P. aeruginosa* PAO1. Therefore the composition of membrane vesicles and their impact in bacterial communities is highly dependent on the circumstances that induces them.

The specific toxic effect of the *S. maltophilia* ciprofloxacin-induced vesicles towards *P. aeruginosa* could have valuable applications in treating *P. aeruginosa* infections. However, the specificity should be further explored on other *P. aeruginosa* strains, and other species, and the role of the membrane vesicles as a carrier should be investigated.

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Discussion

- Chapter 6 -

Conclusions

Nowadays, antibiotic resistance is becoming an important risk for global health. The 'wonder drug' years are over, because misuse and overuse of antibiotic drugs have selected for organisms armed with a range of counter attack mechanisms, leaving certain treatments useless. *Stenotrophomonas maltophilia* is an environmental bacteria, that is loaded with a variety of resistance mechanisms against a whole range of antibiotics (β-lactams, (fluoro)quinolones, aminoglycosides, tetracyclines, sulfonamides), and is increasingly isolated from hospitalized patients and medical devices (Brooke, 2012). Its presence is associated with syndromes like pneumonia (especially in patients with cystic fibrosis), bacteremiae and soft tissue infections, leading to higher mortality rates among these patients.

There is an ongoing debate about the true role of *S. maltophilia* in infection: should it be considered as a bona fide pathogen, or as a mere opportunistic colonizer (Hansen, 2012). Whether the first or last statement is correct (or both), its presence in infectious bacterial communities has an impact on infection start, progress and outcome. *S. maltophilia* is considered an important cystic fibrosis (CF) associated pathogen/colonizer, together with the classic CF pathogens *Pseudomonas aeruginosa*, *Burkholderia cenocepacia* and *Staphylococcus aureus* (Parkins and Floto, 2015). In such polymicrobial communities, inter- and intra-species communication is key for survival. A crucial communication system for *S. maltophilia*, and its co-habitants *P. aeruginosa* and *B. cenocepacia*, is the diffusible signal factor (DSF) fatty acid quorum sensing (QS) system (Ryan and Dow, 2010). *S. maltophilia* produces cis-Δ2-11-methyl-dodecenoic acid (DSF), which can activate its RpfC-RpfG two-component system, eventually leading to c-di-GMP degradation. The cellular pool of c-di-GMP is an important determinant for coordinated responses concerning motility, biofilm formation, and virulence. DSF produced by *S. maltophilia* can also be perceived by *P. aeruginosa* and *B. cenocepacia*, and therefore evoke intra-special responses. For example, DSF led to higher persistence and enhanced antibiotic resistance in *P. aeruginosa* airway biofilms (Twomey et al., 2012). Indeed, while in planktonic growth mode *S. maltophilia* encounters aggressive, competitive behavior of P. aeruginosa, in biofilm mode of growth *S. maltophilia* is capable of modulating the *P. aeruginosa* virulence profile leading to cooperative pathogenicity (Pompilio et al., 2015).

The phenomenon of outer membrane vesicle (OMV) secretion in bacteria has gained a lot of interest during the last years, because of the diverse biological functions that the vesicles perform. OMVs are known to be involved in pathogenesis, virulence, cell-cell communication, biofilm formation, and antibiotic resistance. For now, the OMV biogenesis process is not entirely understood, besides hypothetical models that include the involvement of the number of peptidoglycan (PG)-outer membrane (OM) crosslinks, the outer membrane lipopolysaccharide (LPS) and phospholipid fatty acid composition, and the accumulation of cell material in the periplasm (Schwechheimer and Kuehn, 2015). Furthermore, a link was established between the DSF system and the secretion of OMVs in the plant pathogen *Xylella fastidiosa* (Ionescu et al., 2014), suggesting a possible regulatory role for the QS system. Here the DSF system inhibits the OMV biogenesis process, but stimulates local biofilm formation. A DSF synthase mutant strain produces more OMVs, which most likely block available surfaces for attachment, and eventually this leads to deeper colonization of the host plant. However, in the current work it was shown that *S. maltophilia* produces far more OMVs after stimulating cultures with synthetic DSF (chapter 3, Devos et al., 2015). The stimulatory effect on OMV secretion was only observed at a DSF concentration of 1 mM, while lower concentrations had no effect. The effect of DSF on OMV secretion is therefore concentration-dependent, reflecting a QS-based mechanism. Moreover, the DSF compound produced by *B. cenocepacia*, cis-Δ2-dodecenoic acid (BDSF), also induced OMV biogenesis in *S. maltophilia*, albeit with lower activity compared to DSF (chapter 3, Devos et al., 2015). In *B. cenocepacia*, the BDSF binds to the RpfR receptor and activates its phosphodiesterase activity, leading to c-di-GMP breakdown (Deng et al., 2012). The *S. maltophilia* genome contains a homologous *rpfR* gene, which could explain this effect.

S. maltophilia is particularly known for its intrinsic resistance to β-lactam antibiotics, a consequence of the presence of two chromosomal encoded β-lactamase genes, bla_{L1} and bla_{L2}. These genes are immediately induced after β-lactam exposure. The exposure of *S. maltophilia* to the β-lactam antibiotics penicillin G and imipenem also leads to a strong increase in OMV secretion (chapter 3-4; Devos et al., 2015). The β-lactam-induced reduction in peptidoglycan crosslinks and accumulation of peptidoglycan degradation products is most likely the main cause

of this effect, considering the proposed OMV biogenesis models. Interestingly, a proteomic analysis of the OMV protein cargo revealed the presence of both the L1- and L2-β-lactamase. The packing of β-lactamases in OMVs was already described in *P. aeruginosa*, *Moraxella catarrhalis*, *Staphylococcus aureus* and *Bacteroides* spp. (Ciofu et al., 2000; Schaar et al., 2011; Lee et al., 2013; Stentz et al., 2015). Since the site of action of β-lactams is the periplasm, and the β-lactamases are targeted towards the periplasm (Pradel et al., 2009; Brooke, 2012), it is not entirely surprising that they are picked up during the OMV biogenesis process. What is more intriguing is the secretion of biologically active β-lactamase enzymes into the environment. We showed that both *S. maltophilia* β-lactamases display activity while enclosed in the intact OMVs. The β-lactamase-packed OMVs were capable of hydrolyzing the β-lactam antibiotics imipenem, amoxicillin and ticarcillin, drugs that are frequently used to treat Gram-negative bacterial infections. When these β-lactamase-packed OMVs were administered to cultures of *P. aeruginosa* and *B. cenocepacia*, a drastic increase in antibiotic tolerance was observed for the β-lactams imipenem and ticarcillin. These species are far more susceptible to these drugs than *S. maltophilia*, but could tolerate a 100-fold (or more) increase in drug concentration in the presence of the *S. maltophilia* OMVs. However, we realize that it concerns *in vitro* experiments with purified OMVs, so no intact *S. maltophilia* cells were present. The situation in mixed bacterial cultures could be very different. For example, the presence of *P. aeruginosa* and/or *B. cenocepacia* in the vicinity of *S. maltophilia* could trigger the latter to secrete OMVs with a different cargo, perhaps toxic for other species. Nonetheless, the presence of β-lactamase-packed OMVs in infectious microbial communities could potentially influence treatment outcome, and even facilitate the selection of antibiotic resistance bacteria by lowering the antibiotic concentration to sub-lethal doses.

Next to β-lactams, exposure of *S. maltophilia* to the fluoroquinolone ciprofloxacin also strongly induces OMV secretion. In *P. aeruginosa* it was shown that ciprofloxacin indeed induces OMV secretion (Maredia et al., 2012). The ciprofloxacin-induced OMV secretion was proven to be linked to the cellular SOS response that is evoked as a consequence of DNA damage. When compared to the β-lactam-induced vesicles, ciprofloxacin-induced vesicles show some interesting

differences (chapter 5). Transmission electron microscopy (TEM) revealed a population of vesicles that are considerable larger than the 'classic' OMVs as seen in β-lactam-induced *S. maltophilia* cultures. Furthermore, these larger vesicles often contain long filaments at their surface. Next to vesicle morphology, also the vesicle protein content was analyzed and compared to that of the imipenem-induced (classic) vesicles. What is striking is the enrichment of cytoplasmic and inner membrane proteins in the ciprofloxacin-induced vesicles. The cytoplasmic proteins are mainly represented by ribosomal proteins, but also by proteins involved in protein folding- and DNA damage- related stress. The toxic effect of ciprofloxacin leads to the accumulation of misfolded proteins, which triggers the production of chaperones and heat shock proteins (GroEL-GroES complex, DnaK, HtpG, SurA). DnaK and GroEL were already shown to be induced in response to antibiotic stress in *Acinetobacter baumannii* (Cardoso et al., 2010). Whether the GroEL protein complex in *S. maltophilia* is associated with the secreted membrane vesicles is not sure. The electron microscopy images show the abundant co-purification of protein complexes next to the vesicles, from which the shape resembles that of GroEL. Furthermore, the ciprofloxacin-induced vesicles contained more inner membrane proteins, with the striking presence of several ATP synthase complex subunits (α-, β-, ε-, γ- and b-subuit), almost entirely absent in the imipenem-induced vesicles. This raised the question whether the inner membrane is part of the secreted vesicles, constituting so called outer-inner membrane vesicles (OIMV) recently described as a new type of bacterial membrane vesicles (Pérez-Cruz et al., 2015). This was confirmed with cryo-TEM, where it was shown that most of the larger ciprofloxacin-induced vesicles contained a double membrane layer. Unfortunately, the exact mechanism how ciprofloxacin induces vesicle secretion is not known. The accumulation of misfolded protein in the periplasm could be an explanation. However, in *P. aeruginosa* it was found that vesiculation was increased through the synthesis of B-band LPS induced by oxidative stress (Macdonald and Kuehn, 2013). B-band LPS contains longer and highly charged O antigen, capable of causing curvature in the outer membrane. Ciprofloxacin-induced oxidative stress has been demonstrated in *Escherichia coli*, as well as in *P. aeruginosa* (Goswami et al., 2006; Jensen et al., 2014). The reactive oxygen species (ROS) superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are believed to amplify the bactericidal effect of ciprofloxacin. The presence of several superoxide dismutases (*sodA*, *sodC1*, *sodC2*) and a subunit from the alkyl

hydroperoxide reductase (*ahpC*) in the membrane vesicles agrees with this premise. Though, since these proteins are also found in the imipenem-induced vesicles, this response is probably not specific for ciprofloxacin.

An interesting finding in the ciprofloxacin vesicles is the identification of a group of phage related proteins that are not found in the imipenem-induced vesicles (chapter 5). The *S. maltophilia* genome contains a cluster of prophage genes, similar to that of the P2 phage. The major tail sheath protein that was identified was previously described in *S. maltophilia* strain P28 as the phage tail-like maltocin P28 (Liu et al., 2013). The phage tail major structural components, the tail sheath and tail tube proteins, compose a contractile and rigid phage tail filament, resembling the pyocin phage tail particles found in *P. aeruginosa* (Michel-Briand and Baysse, 2002). Maltocin P28 is a bacteriocin showing bactericidal activity against many *S. maltophilia* strains. As mentioned before, the ciprofloxacin-induced vesicles carry filament structures at the surface, and this now raised the question if these could be phage tail filaments. Since these potential phage filaments are thus attached to the membrane vesicles, the purified intact vesicles were tested for bactericidal activity against *S. maltophilia* itself (strain 44/98), but also against the frequent cohabitating species *P. aeruginosa* (strain PAO1) and *B. cenocepacia* (type strain). Remarkably, the phage filament carrying vesicles only show a bactericidal effect towards *P. aeruginosa* PAO1. What aspect determines the species specificity is unclear, but worthwhile to find out. Furthermore, the vesicles do not lose their toxicity after treating the intact vesicles with proteinase K (up to 1 mg/ml), Triton X-100, or both. This means that the toxic entity is highly resistant to protease treatment, and independent from the intact vesicles as carriers.

Other abundant cargo constituents identified in *S. maltophilia* membrane vesicles, are two Ax21 homologs (chapter 3). Ax21 is an OMV associated outer membrane β-barrel protein proven to be involved in motility and biofilm formation (Park et al., 2014). Studies have shown that Ax21 expression is regulated by the DSF QS system (Qian et al., 2013). The stimulation of *S. maltophilia* cultures with imipenem, and DSF and BDSF, all stimulated the OMV-mediated secretion of both Ax21 homologs. However, the results point to a stimuli-dependent difference in the proportion

of the homologs secreted in the vesicles. And, although BDSF induces far less vesicle secretion than DSF, the overall vesicular Ax21 content is more or less the same. Unfortunately, the exact function of the Ax21 protein remains unclear. Considering its structure resembles that of protein translocators for autotransporters (e.g. OmpF), it is likely to be involved in the translocation of virulence factors. Appropriate knockout mutants should be generated to identify potential Ax21 mediated translocated proteins.

Besides the Ax21 protein, OMVs on their own too seem to influence biofilm formation. However, studies report on both positive and negative effects of OMVs on biofilm formation (Yonezawa et al., 2009; Ho et al., 2015; Ionescu et al., 2014). β-lactam-induced OMVs from *S. maltophilia* demonstrated an inhibitory effect on its own biofilm formation, but stimulated biofilm formation in *P. aeruginosa* and *B. cenocepacia* cultures (chapter 4). When the cultures were grown in the growth chambers pre-incubated with OMVs, followed by washing the chambers, the same effect was observed. This means that the OMVs adhere to the growth chamber surfaces, and then either inhibit (by blocking) or stimulate (by interaction) cell attachment and subsequent biofilm formation. The molecular mechanism behind this phenomenon is still to be elucidated. Investigating how the OMVs establish intra- and inter-species interactions would be helpful in understanding the roles of OMVs in polymicrobial communities, under different circumstances.

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- Chapter 7 -

Future perspectives

Allthough there are several models describing the process of OMV biogenesis, the knowledge is still incoherent and incomplete (Schwechheimer and Kuehn, 2015). How do cells perceive a certain signal and then respond by secreting membrane vesicles? As it is often the case in system biology, OMV biogenesis is probably complex and dependent on several factors. This work showed significant differences in the *Stenotrophomonas maltophilia* OMV secretion process when comparing the β-lactam imipenem and the fluoroquinolone ciprofloxacin as stimulatory agents. While the increased OMV secretion during β-lactam stress fits the proposed models quite well (accumulation of peptidoglycan degradation products, reduced number of cell wall crosslinks), the link between ciprofloxacin-induced stress and vesicle secretion is not completely understood. Oxidative stress and the SOS response have been proposed as potential inducers, but this needs further investigation (Maredia et al., 2012). Also it was observed that ciprofloxacin leads to the secretion of two different types of vesicles, OMVs and outer-inner membrane vesicles (OIMVs), pointing to parallel vesiculation processes. Moreover, the diffusible signal factor (DSF) system influenced OMV secretion as well. The *S. maltophilia* DSF molecule cis-Δ2-11-methyl-dodecenoic acid and the *Burkholderia cenocepacia* DSF molecule cis-Δ2-dodecenoic acid both had a stimulatory effect on OMV secretion in *S. maltophilia*. In contrast, in the closely related plant pathogen *Xylella fastidiosa* the DSF system showed inhibitory effects on OMV secretion. How this DSF quorum sensing system is involved in OMV biogenesis is still to be determined. A time-kinetic, multi-level system biology analysis approach (transcriptomics, proteomics, lipidomics, metabolomics) during the OMV production will determine potential important players and regulatory mechanisms in the OMV biogenesis process.

The current work characterized OMVs *in vitro*, with pure cultures under controlled conditions. *S. maltophilia* is often part of polymicrobial communities causing chronic pulmonary infections in patient with cystic fibrosis (CF). Therefore it would be interesting to analyze OMVs in mixed-species cultures in artificial sputum medium (ASM) mimicking CF sputum, or actual sputum, whether or not exposed to antibiotics. This will broaden the knowledge about the beneficial and/or harmfull roles of OMVs in polymicrobial communities, depending on different antibiotic treatments. Stand-alone OMV functions like extracellular degradation of antibiotics by enzyme

cargo is evident, but the delivery of the vesicle content to other cells (intra- or inter-species) is less straightforward. This would imply the docking of vesicles on target cells, fusing with the outer membrane, and the delivery of the functional cargo into the cell. However, the ability of *S. maltophilia* OMVs to deliver functional resistance proteins or genes to other cells still needs to be proven.

The ability of *S. maltophilia* to grow as a biofilm is an important trait contributing to the survival of the species. OMVs are believed to play an important role in the biofilm formation process. This work showed that β-lactam-induced *S. maltophilia* OMVs have an inhibitory effect on its transition to biofilm mode of growth, presumably through the blocking of available surface areas. Meanwhile these same OMVs stimulated biofilm formation in *Pseudomonas aeruginosa* and *B. cenocepacia* cultures. The molecular mechanisms behind this phenomenon are unknown, and an interesting topic for future research. Also related to biofilm formation, two Ax21 homologues were identified in *S. maltophilia*, showing abundant OMV-associated secretion after antibiotic treatment. Ax21 was shown to be important for virulence and biofilm formation in *S. maltophilia* and closely related species, but its actual function is still not clear. The outer membrane protein is structurally homologues to the transporter domains of autotransporters, and could therefore be involved in the translocation of virulence factors. Secretome analysis of wild type versus Ax21 knock-out strains could potentially clarify the Ax21 function.

Finally, ciprofloxacin-induced membrane vesicles appeared to be highly toxic for the *P. aeruginosa* strain PAO1. The cell stress evoked by ciprofloxacin led to the expression of a cluster of phage-related genes, and the subsequent OMV-associated secretion of phage tail bacteriocins (tailocins). In this work, the bactericidal effect of these vesicles was specific towards *P. aeruginosa* (strain PAO1), and not towards *S. maltophilia* 44/98 itself or the *B. cenocepacia* type strain. The spectrum of activity should be further explored on a range of other *P. aeruginosa* strains, and other species. The bactericidal effect of the *S. maltophilia* tailocins can potentially be exploited for treating *P. aeruginosa* infections. In *S. maltophilia* these tailocins are attached to the surface of membrane vesicles, but the reason for this is unclear. The use of vesicles as carriers may have

different advantages: more efficient delivery of toxins to the target cells, long distance transport, or improved penetration in biofilms. Membrane vesicles, or artificial liposomes, show great potential in the delivery of antimicrobials to biofilms (Forier et al., 2014), and are already used in vaccine development (Acevedo et al., 2014). For example, membrane vesicle-based vaccines exist for meningitis, cholera, pertussis and tuberculosis. The exact mechanism of action of the *S. maltophilia* tailocin, the molecular basis of specificity and the role of membrane vesicles as carriers should be clarified if it would be considered as a potential antibacterial drug.

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Addendum

A1. Material and methods for the time-kinetic, quantitative proteome study

Materials

Urea was obtained from GE Healthcare (Diegem, Belgium). 'Complete mini' EDTA-free protease inhibitor mix was purchased from Roche Diagnostics (Vilvoorde, Belgium). Rapigest detergent and the rabbit glycogen phosphorylase B standard peptide mixture were from Waters Corporation (Milford, MA, US). Sequencing grade modified trypsin was obtained from Promega (Madison, WI, US), while mass spectrometry grade lysyl endopeptidase was from Wako Chemicals (GmbH, Neuss, Germany). ULC-MS grade water, acetonitrile (ACN) and formic acid was procured from Biosolve (Valkenswaard, The Netherlands). Imipenem was kindly donated by Prof. M. Galleni (CIP, University of Liège, Belgium). Other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, US).

Bacterial cell culture

The imipenem-resistant *S. maltophilia* strain 44/98 (BCCM/LMG bacteria collection, LMG 26824) was isolated at the Clinical Microbiology Unit of the Varese University hospital in Italy. Cells were grown aerobically overnight as two separate cultures until the stationary phase. The cell suspensions were then separately diluted 10-fold in 300 mL of fresh Luria Broth (LB) medium, and allowed to grow further until they reached the mid-exponential growth phase (OD_{600nm} = 0.65-0.75). From these two culture flasks, samples were harvested by centrifugation at 2,500 x g for 5 min (Time 0). The remaining cultures were stimulated with 25 µg/mL imipenem. Samples were also harvested 30 minutes, 1 h, 2 h and finally 3 h after the imipenem challenge (Time 0.5, 1, 2 and 3 respectively, each in duplicate).

Sample preparation for LCMS^E

The pellets were resuspended in a solution containing 6 M urea, 2 M thiourea, 0.2% Rapigest, and an EDTA-free protease inhibitor mixture in 50 mM ammonium bicarbonate. The cells were disrupted with acid-washed glass beads (diameter of 1-1.25 mm) for 180 s in a Precellys 24 instrument (Bertin Technologies, Orléans, France). The protein solutions were cleaned-up and concentrated by acetone precipitation, followed by resuspension and rinsing on a 10 kDa cut-off spin column (Ultracel Amicon, Millipore, MA, US) with 0.2% Rapigest, 1 mM DTT and 50 mM ammonium bicarbonate as the buffer. The protein concentration was assessed using the Coomassie Plus Bradford™ Assay kit (Thermo Scientific, San Jose, CA, US). Ten µg of each protein extract was heated at 80 °C for 15 min, reduced with 2.5 mM DTT for 45 min at 60 °C and subsequently alkylated with 7.5 mM iodoacetamide at ambient temperature for 30 min. The proteins were first digested with 1:50 (w/w) lysyl endopeptidase for 3 h at ambient temperature, followed by trypsin digestion (1:50 w/w) overnight at 37 °C. The Rapigest detergent was hydrolyzed by the addition of formic acid to the solution, and removed by centrifugation. Each sample was diluted with a glycogen phosphorylase B

standard peptide mixture and 0.1% formic acid in water to give a final protein concentration of 0.5 µg/µl per sample and 50 fmol/µl phosphorylase B.

LCMS^E analysis

The peptide mixtures were separated on a NanoAcquity UPLC® system (Waters Corporation) using a Symmetry® C18 trapping column (180 µm x 20 mm, 5 µm) and a BEH™ C18 analytical column (75 µm x 250 mm, 1.7 µm) at 40 °C. Solvent A and B were composed of 0.1% formic acid in water and 0.1% formic acid in ACN, respectively. Each sample (0.5 µg of total protein and 50 fmol of phosphorylase B) was loaded onto the trapping column with 0.1% solvent B for 1 min at 15 µL/min and eluted at 250 nL/min by increasing the organic solvent concentration from 3-40% B over 90 min. Analyses were performed in quadruplicate. The eluting peptides were directly ionized and analyzed with a SYNAPT™ HDMS using a PicoTip Emitter from New Objective (uncoated silicaTip™ 10 +/- 1 µm, Woburn, MA, US). The time-of-flight (TOF) analyzer was externally calibrated with MS/MS fragments of human [glu^1]-fibrinopeptide B (GFP) from *m/z* 72 to 1285, and the data was corrected post-acquisition using the monoisotopic mass of the doubly charged precursor of GFP (*m/z* 785.8426), which was measured with a collision energy of 6.0 V and sampling cone voltage of 45 V (lockmass correction). The GFP was delivered at 500 fmol/µL to the mass analyzer by a NanoLockSpray interface using the auxiliary pump of the NanoAcquity system at a flow rate of 300 nL/min. The reference sprayer was sampled every 30 s. Accurate mass data were collected in a data independent positive mode of acquisition (MS^E) from 15 to 120 minutes by alternating between low (5 V) and high (ramping from 15 to 35 V) energy scan functions. The spectral acquisition scan rate was 0.48 s with a 0.1 s inter-scan delay. The selected *m/z* range was 125 to 2000 Da. The capillary voltage was set to 3.0 kV, the sampling cone voltage was 26 V and the extraction cone voltage on 2.65 V. The source temperature was set on 65 °C.

Identification of proteins

The LCMS^E data were processed using the ProteinLynx Global SERVER™ v2.5 (PLGS, Waters Corporation). In brief, lockmass-corrected spectra (0.250 Da window allowed) were automatically centroided, deisotoped and charge-state reduced to produce a single monoisotopic peak for each peptide and associated fragment ion. The correlation of a precursor and a potential fragment ion was achieved by means of time alignment, in the first instance. The following parameters were used for the data processing in PLGS: the chromatographic peak width, the TOF resolution and retention time window, which were determined automatically by the software, and the low energy, high energy, and intensity thresholds, which were set to 250, 100 and 1500 counts respectively. A database containing 4369 protein entries from the closely related *Stenotrophomonas maltophilia* K279a (Uniprot website, June 2011), phosphorylase B (spiked into each sample) and potential contaminating proteins, as well as the randomized entries of all the proteins was interrogated by PLGS. The precursor and fragment ion tolerance were determined automatically. The default

protein identification criteria used included a maximal protein mass of 500,000 Da, a detection of minimal 3 fragment ions per peptide, minimal 7 fragment ions per protein and minimal 1 peptide per protein. Fixed modification of carbamidomethyl-C and the variable modifications included acetylation (N-terminus), deamidation (N/Q) and oxidation of the methionines were selected. Maximally two missed cleavages and a false positive rate of 4% was allowed. The quality of the LCMS^{E} runs was examined with MassLynx v 4.1 (Waters Corporation), IBM® SPSS Statistics v 19, and Excel.

Label-free quantitative analysis with Expression^{E} from PLGS

A relative quantification analysis of the protein abundances at the different time points, before and after the antibiotic challenge, was performed using the Expression^{E} software integrated in PLGS v.2.5. The algorithm uses the weighted sum of the peak intensities of all peptides for the relative quantification of the proteins. The contribution of a peptide intensity in the protein quantification is based on the uniformity of the intensities of this peptide in the technical replicates, as well as the peptide identification score. Protein ratios were normalized to the intensity of the dominant background of proteins showing no change in abundance between the different time collections. The proteins had to be identified in at least 2 technical replicates, with a score of 150 and a probability of 95%, as well as a confidence limit of maximally 2.5. The significance level of regulation was set at 30% fold change, corresponding to an average relative fold change of -0.3 and 0.3 on a natural log scale. This is 2-3 times higher than the estimated error on the intensity measurement. The results were exported to Excel and IBM® SPSS Statistics v 19 for further analysis.

A2. OMV proteomics on imipenem-induced OMVs

Protein identification list of the 2D-LCMS OMV proteome analysis (identified in minimum 2/3 replicate runs)

Entry	Protein names	Gene names
B2FIV0	10 kDa chaperonin (GroES protein) (Protein Cpn10)	groS groES Smlt4215
B2FNP5	30S ribosomal protein S1	rpsA Smlt2043
B2FN87	30S ribosomal protein S15	rpsO Smlt3386
B2FIA9	30S ribosomal protein S2	rpsB Smlt1507
B2FQ51	30S ribosomal protein S3	rpsC Smlt0912
B2FJU4	50S ribosomal protein L13	rplM Smlt4302
B2FQ52	50S ribosomal protein L16	rplP Smlt0913
B2FQK9	50S ribosomal protein L17	rplQ Smlt0933
B2FQ48	50S ribosomal protein L2	rplB Smlt0909
B2FN76	50S ribosomal protein L20	rplT Smlt3375
B2FTD1	50S ribosomal protein L21	rplU Smlt1278
B2FQ20	50S ribosomal protein L25 (General stress protein CTC)	rplY ctc Smlt0876
B2FQJ6	50S ribosomal protein L5	rplE Smlt0918
B2FQ37	50S ribosomal protein L7/L12	rplL Smlt0897
B2FKJ7	50S ribosomal protein L9	rplI Smlt3148
B2FIU9	60 kDa chaperonin (GroEL protein) (Protein Cpn60)	groL groEL Smlt4214
B2FT48	Adenylate kinase (AK) (EC 2.7.4.3) (ATP-AMP transphosphorylase) (ATP:AMP phosphotransferase) (Adenylate monophosphate kinase)	adk Smlt3886
B2FKK2	Asparagine--tRNA ligase (EC 6.1.1.22) (Asparaginyl-tRNA synthetase)	asnS Smlt3154
B2FHZ0	ATP synthase subunit alpha (EC 3.6.3.14) (ATP synthase F1 sector subunit alpha) (F-ATPase subunit alpha)	atpA Smlt4113
B2FHY8	ATP synthase subunit beta (EC 3.6.3.14) (ATP synthase F1 sector subunit beta) (F-ATPase subunit beta)	atpD Smlt4111
B2FUV6	ATP-dependent protease ATPase subunit HslU (Unfoldase HslU)	hslU Smlt4075
B2FJU0	Bacterioferritin (EC 1.16.3.1)	bfr Smlt4297
B2FNP1	Cell division protein ftsA	ftsA Smlt0759
B2FTA6	Cell division topological specificity factor	minE Smlt1250
B2FMY5	Chaperone protein DnaK (HSP70) (Heat shock 70 kDa protein) (Heat shock protein 70)	dnaK Smlt1992
B2FLW2	Chaperone protein HtpG (Heat shock protein HtpG) (High temperature protein G)	htpG Smlt1809
B2FPG7	Chaperone SurA (Peptidyl-prolyl cis-trans isomerase SurA) (Rotamase SurA)	surA Smlt0820
B2FSI7	Citrate synthase	gltA Smlt3835
B2FI05	Conserved hypothetical exported protein	Smlt4128
B2FJC6	Conserved hypothetical exported protein	Smlt1634
B2FKV7	Conserved hypothetical exported protein	Smlt0483
B2FL62	Conserved hypothetical exported protein	Smlt1774
B2FLW5	Conserved hypothetical exported protein	Smlt1812
B2FMI3	Conserved hypothetical exported protein	Smlt4500
B2FN54	Conserved hypothetical exported protein	Smlt3351
B2FNH0	Conserved hypothetical exported protein	Smlt4628
B2FP55	Conserved hypothetical exported protein	Smlt4642
B2FQN8	Conserved hypothetical exported protein	Smlt0965
B2FQX4	Conserved hypothetical exported protein	Smlt2334
B2FQY5	Conserved hypothetical exported protein	Smlt2345
B2FR42	Conserved hypothetical exported protein	Smlt3680
B2FR48	Conserved hypothetical exported protein	Smlt3686
B2FRX3	Conserved hypothetical exported protein	Smlt1121
B2FRX4	Conserved hypothetical exported protein	Smlt1122

B2FRX9	Conserved hypothetical exported protein	Smlt1127
B2FRZ2	Conserved hypothetical exported protein	Smlt1140
B2FSE9	Conserved hypothetical exported protein	Smlt3796
B2FTB2	Conserved hypothetical exported protein	Smlt1256
B2FN59	Conserved hypothetical repetitive protein	Smlt3358
B2FT86	Conserved hypothetical TPR repeat family protein	Smlt0008
B2FLD3	Dihydrolipoyl dehydrogenase (EC 1.8.1.4)	odhL Smlt3199
B2FQK8	DNA-directed RNA polymerase subunit alpha (RNAP subunit alpha) (EC 2.7.7.6) (RNA polymerase subunit alpha) (Transcriptase subunit alpha)	rpoA Smlt0931
B2FQ39	DNA-directed RNA polymerase subunit beta' (RNAP subunit beta') (EC 2.7.7.6) (RNA polymerase subunit beta') (Transcriptase subunit beta')	rpoC Smlt0899
B2FQ38	DNA-directed RNA polymerase subunit beta (RNAP subunit beta) (EC 2.7.7.6) (RNA polymerase subunit beta) (Transcriptase subunit beta)	rpoB Smlt0898
B2FQ42	Elongation factor G (EF-G)	fusA Smlt0903
B2FIA8	Elongation factor Ts (EF-Ts)	tsf Smlt1506
B2FQ31	Elongation factor Tu (EF-Tu)	tufB tuf Smlt0890 Smlt0904
B2FK88	Enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase) (2-phosphoglycerate dehydratase)	eno Smlt1715
B2FU50	Glucans biosynthesis protein D	opgD Smlt0091
B2FQR4	Lon protease (EC 3.4.21.53) (ATP-dependent protease La)	lon Smlt0991
B2FPG8	LPS-assembly protein LptD (Organic solvent tolerance protein)	lptD ostA Smlt0821
B2FQL8	Malate dehydrogenase (EC 1.1.1.37)	mdh Smlt0944
B2FNQ5	Nucleoside diphosphate kinase (NDK) (NDP kinase) (EC 2.7.4.6) (Nucleoside-2-P kinase)	ndk Smlt2054
B2FIA0	Outer membrane protein assembly factor BamA	bamA Smlt1498
B2FNR0	Outer membrane protein assembly factor BamB	bamB Smlt2059
B2FRR9	Outer membrane protein assembly factor BamD	comL bamD Smlt3748
B2FMX8	Outer membrane protein assembly factor BamE	bamE Smlt1985
B2FQY6	Outer-membrane lipoprotein carrier protein	Smlt2346
B2FQ17	Outer-membrane lipoprotein LolB	lolB Smlt0873
B2FLB8	Peptidyl-prolyl cis-trans isomerase	Smlt3182
B2FIF8	Peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8)	Smlt1559
B2FN86	Polyribonucleotide nucleotidyltransferase (EC 2.7.7.8) (Polynucleotide phosphorylase) (PNPase)	pnp Smlt3385
B2FSQ8	Probable malate:quinone oxidoreductase (EC 1.1.5.4) (MQO) (Malate dehydrogenase [quinone])	mqo Smlt1234
B2FMY4	Protein GrpE (HSP-70 cofactor)	grpE Smlt1991
B2FRM9	Protein TolB	tolB Smlt3704
B2FPB2	Protein translocase subunit SecA	secA Smlt0764
B2FHD7	Protein-export protein SecB	secB Smlt0171
B2FKN2	Putative 4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27)	Smlt4330
B2FP85	Putative ABC transporter toluene tolerance exported protein	Smlt4673
B2FT31	Putative ACR family protein	Smlt3869
B2FP33	Putative alkaline phosphatase	Smlt3463
B2FPY7	Putative alkyl hydroperoxide reductase subunit c (EC 1.11.1.15)	ahpC Smlt0841
B2FJX9	Putative aminopeptidase	Smlt0418
B2FL11	Putative aminopeptidase	Smlt0541
B2FQE3	Putative angiotensin-converting enzyme like peptidyl dipeptidase protein	Smlt3574
B2FR62	Putative autotransporter	Smlt1001
B2FPV5	Putative autotransporter subtilisin-like protease	sphB Smlt3524
B2FRP5	Putative beta-lactamase	Smlt3722
B2FT88	Putative biopolymer transport exbB protein	exbB1 Smlt0010
B2FT89	Putative biopolymer transport ExbD1 protein	exbD1 Smlt0011
B2FPM6	Putative calcineurin phosphoesterase	Smlt2170
B2FJY2	Putative carboxy-terminal processing protease (EC 3.4.21.102)	ctpA Smlt0421

B2FK12	Putative dehydrogenase	Smlt0451
B2FUE8	Putative diaminobutyrate--2-oxoglutarate aminotransferase (EC 2.6.1.76)	dat Smlt1415
B2FSF8	Putative endonuclease P1 (EC 3.1.30.1)	Smlt3806
B2FP18	Putative endopeptidase O (EC 3.4.24.-)	pepO Smlt3447
B2FMI6	Putative exported dipeptidyl peptidase IV	Smlt4503
B2FP20	Putative exported endopeptidase	Smlt3450
B2FI03	Putative exported lipoprotein	Smlt4126
B2FTA3	Putative exported peptidase (EC 3.4.14.-)	Smlt1246
B2FUT3	Putative exported rare lipoprotein A	rlpA Smlt4051
B2FUS7	Putative exported tail-specific protease (EC 3.4.21.102)	Smlt4045
B2FJY4	Putative fatty acid transport system, membrane protein	Smlt0423
B2FR50	Putative ferritin DPS-family DNA binding protein	Smlt3688
B2FNJ3	Putative fimbria adhesin protein	Smlt0709
B2FNJ0	Putative fimbrial adhesin protein	smf-1 Smlt0706
B2FNQ7	Putative fimbrial biogenesis protein	Smlt2056
B2FIR8	Putative fimbrial protein (Pilin)	Smlt4182
B2FQU5	Putative flagellin	fliC Smlt2304
B2FQU6	Putative flagellin	flaA Smlt2305
B2FST5	Putative glucan 1,4-beta-glucosidase	Smlt2569
B2FP87	Putative intercellular spreading VacJ lipoprotein	Smlt4675
B2FRZ9	Putative iron transport receptor protein	Smlt1148
B2FHT9	Putative iron transporter	Smlt2858
B2FJR8	Putative isocitrate dehydrogenase [NADP] (EC 1.1.1.42)	icd Smlt4273
B2FQQ5	Putative isocitrate/isopropylmalate dehydrogenase	Smlt0982
B2FP30	Putative lipoprotein	Smlt3460
B2FUS6	Putative lipoprotein	Smlt4044
B2FU57	Putative lipoprotein E (Outer membrane protein p4)	hel Smlt0098
B2FNM5	Putative LppC family lipoprotein	Smlt0742
B2FP06	Putative membrane-bound lytic murein transglycosylase d (EC 3.2.1.-)	mltD Smlt3434
B2FTM1	Putative metallo-beta-lactamase l1 (Beta-lactamase type ii) (Ec 3.5.2.6) (Penicillinase) (EC 3.5.2.6)	Smlt2667
B2FRQ3	Putative MltA scaffolding protein	Smlt3731
B2FH96	Putative modulator of DNA gyrase	Smlt0129
B2FQU7	Putative motility flagellin protein	Smlt2306
B2FUT4	Putative murein hydrolase (EC 3.2.1.-)	mltB Smlt4052
B2FHC0	Putative N-acetylmuramoyl-L-alanine amidase	Smlt0154
B2FJB1	Putative oar family adhesion protein	Smlt1619
B2FRW6	Putative OstA family protein	Smlt1114
B2FLE9	Putative outer membrane antigen lipoprotein	Smlt3215
B2FLE4	Putative outer membrane antigen protein	Smlt3210
B2FLY6	Putative outer membrane efflux protein	smeX Smlt1833
B2FSC8	Putative outer membrane esterase	Smlt3773
B2FLX9	Putative outer membrane lipoprotein	Smlt1826
B2FPV8	Putative outer membrane lipoprotein	Smlt3527
B2FR78	Putative outer membrane lipoprotein	Smlt1018
B2FI00	Putative outer membrane Omp family protein	Smlt4123
B2FSF7	Putative outer membrane Omp family protein	Smlt3805
B2FLU3	Putative outer membrane protein	Smlt0613
B2FLU4	Putative outer membrane protein	Smlt0614
B2FQM8	Putative outer membrane protein	Smlt0955
B2FTS5	Putative outer membrane protein	tolC Smlt3928
B2FSS7	Putative outer membrane regulator of pathogenicity factors protein	rpfN Smlt2559
B2FNJ2	Putative outer membrane usher protein mrkc	mrkC Smlt0708
B2FU40	Putative patatin-like phospholipase	Smlt0080
B2FUT2	Putative penicillin-binding protein (EC 3.4.16.4)	dacC Smlt4050

B2FR43	Putative penicillin-binding protein 1B (EC 2.4.1.129)	mrcB Smlt3681
B2FHG8	Putative peptidase	Smlt0203
B2FJ20	Putative peptidase	Smlt0348
B2FT85	Putative peptidase	Smlt0007
B2FUU5	Putative PEPTIDASE	Smlt4064
B2FRM8	Putative peptidoglycan-associated lipoprotein	Smlt3703
B2FLG2	Putative peptidyl dipeptidase/oligopeptidase	Smlt3229
B2FI43	Putative peptidyl-dipeptidase Dcp (Dipeptidyl carboxypeptidase) (EC 3.4.15.5)	dcp Smlt0223
B2FR55	Putative peptidyl-prolyl cis-trans isomerase	Smlt0993
B2FU43	Putative phosphatase	Smlt0084
B2FTU3	Putative phosphate selective porin	Smlt3950
B2FHJ5	Putative phosphodiesterase-nucleotide pyrophosphatase	Smlt1449
B2FNJ1	Putative pili chaperone protein	Smlt0707
B2FTT7	Putative porin P (Outer membrane protein d1)	oprP Smlt3943
B2FJ52	Putative protease	Smlt0381
B2FUA1	Putative quinol oxidase subunit 1	qoxB Smlt1361
B2FPR6	Putative rare lipoprotein B family protein	Smlt3484
B2FUU0	Putative rod shape-determining protein	mreB Smlt4059
B2FPY1	Putative signal peptidase I (EC 3.4.21.89)	lepB Smlt3551
B2FK97	Putative subfamily M23B unassigned peptidase	Smlt1724
B2FQC5	Putative subfamily S1C unassigned peptidase	Smlt3553
B2FTY8	Putative thiol:disulfide interchange protein	Smlt3994
B2FQ11	Putative thioredoxin electron transport related protein	Smlt0866
B2FRN0	Putative TolA transmembrane protein	tolA Smlt3705
B2FRN2	Putative TolQ transport transmembrane protein	tolQ Smlt3707
B2FRN1	Putative TolR-related protein	tolR Smlt3706
B2FN47	Putative TonB dependent receptor	Smlt3340
B2FP16	Putative TonB dependent receptor	Smlt3444
B2FR08	Putative TonB dependent receptor	Smlt3645
B2FRR1	Putative TonB dependent receptor	Smlt3740
B2FST2	Putative TonB dependent receptor	Smlt2566
B2FT66	Putative TonB dependent receptor	Smlt3905
B2FJ30	Putative TonB dependent receptor protein	Smlt0359
B2FKT0	Putative TonB dependent receptor protein	Smlt4387
B2FM99	Putative TonB dependent receptor protein	Smlt3254
B2FP17	Putative TonB dependent receptor protein	Smlt3446
B2FU42	Putative TonB dependent receptor protein	Smlt0083
B2FHH2	Putative TonB dependent siderophore receptor	Smlt1426
B2FKE5	Putative TonB domain protein	Smlt3093
B2FPN5	Putative TonB-dependent outer membrane receptor protein	Smlt2179
B2FUR1	Putative TonB-dependent outer membrane receptor protein	Smlt4026
B2FRC4	Putative TonB-dependent receptor	Smlt1067
B2FRM7	Putative TPR repeat exported protein	Smlt3702
B2FUP4	Putative transglycosylase	Smlt4007
B2FP62	Putative transglycosylase protein	Smlt4650
B2FL08	Putative transmembrane anchor protein	Smlt0538
B2FLQ3	Putative transmembrane anchor protein	Smlt0569
B2FHD1	Putative transmembrane HemY porphyrin biosynthesis protein	Smlt0165
B2FHE8	Putative transmembrane protein	Smlt0182
B2FN01	Putative transmembrane protein	Smlt2010
B2FQ16	Putative transmembrane protein	Smlt0872
B2FIV3	Putative transmembrane Thiol:disulfide Interchange Protein	Smlt4218
B2FLR8	Putative vitamin B12 receptor protein	Smlt0585
B2FJ75	Putrescine-binding periplasmic protein	potF Smlt1581
B2FM92	Ribonuclease E (RNase E) (EC 3.1.26.12)	rnE rne Smlt3247

B2FTA7	Site-determining protein	minD Smlt1251
B2FRS2	Succinyl-CoA ligase [ADP-forming] subunit alpha (EC 6.2.1.5)	sucD Smlt3752
B2FHC6	Superoxide dismutase [Cu-Zn] (EC 1.15.1.1)	sodC1 Smlt0160
B2FHC7	Superoxide dismutase [Cu-Zn] (EC 1.15.1.1)	sodC2 Smlt0161
B2FTY7	Thiol:disulfide interchange protein	dsbA Smlt3993
B2FQ33	Transcription termination/antitermination protein NusG	nusG Smlt0892
B2FQR1	Trigger factor (TF) (EC 5.2.1.8) (PPIase)	tig Smlt0988
B2FHB5	Uncharacterized protein	Smlt0149
B2FHE7	Uncharacterized protein	Smlt0181
B2FHF0	Uncharacterized protein	Smlt0184
B2FHG9	Uncharacterized protein	Smlt0204
B2FHL9	Uncharacterized protein	Smlt1474
B2FHN9	Uncharacterized protein	Smlt2799
B2FHZ6	Uncharacterized protein	Smlt4119
B2FII7	Uncharacterized protein	Smlt2905
B2FIM5	Uncharacterized protein	Smlt2944
B2FJR9	Uncharacterized protein	Smlt4275
B2FJV0	Uncharacterized protein	Smlt0387
B2FJV9	Uncharacterized protein	Smlt0397
B2FJY0	Uncharacterized protein	Smlt0419
B2FL09	Uncharacterized protein	Smlt0539
B2FLG5	Uncharacterized protein	Smlt3232
B2FME4	Uncharacterized protein	Smlt3304
B2FND4	Uncharacterized protein	Smlt4590
B2FND7	Uncharacterized protein	Smlt4593
B2FNK9	Uncharacterized protein	Smlt0725
B2FQ57	Uncharacterized protein	Smlt2204
B2FQN1	Uncharacterized protein	Smlt0958
B2FQN3	Uncharacterized protein	Smlt0960
B2FQN5	Uncharacterized protein	Smlt0962
B2FTS4	Uncharacterized protein	pcm Smlt3927
B2FUD6	Uncharacterized protein	Smlt1403
B2FUU4	Uncharacterized protein	Smlt4063

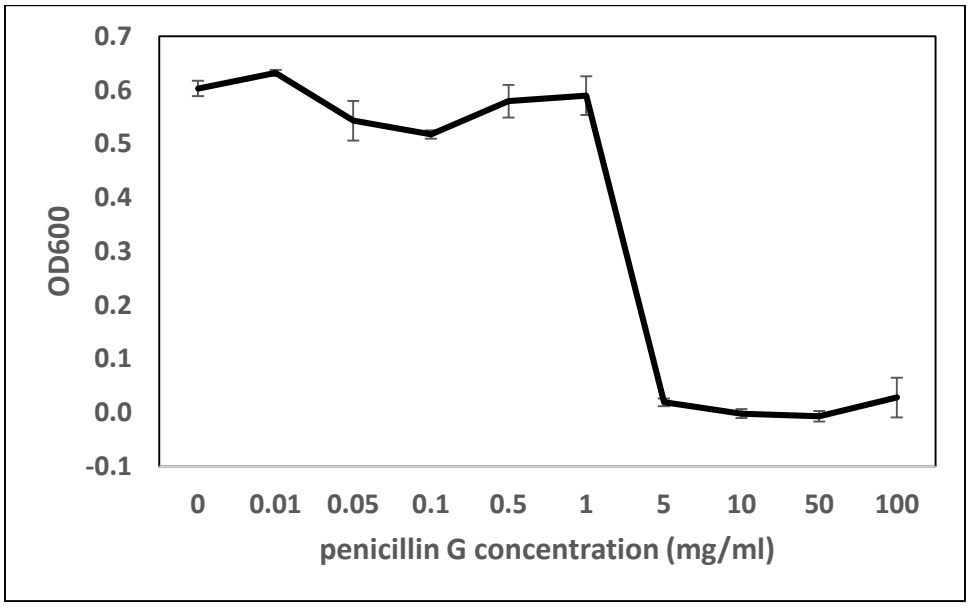
A3. MRM target peptide transition list

Protein	Peptide precursor	Fragment ion	Precursor m/z (Da)	Fragment ion m/z (Da)	Dwell time (msec)	DP (V)	CE (V)
Smlt0184	IGAGYNYGIAPNTDLVAR (2+)	y10 (+)	932.98	1069.60	100	99.1	51.6
		y9 (+)	932.98	956.52	100	99.1	51.6
		y8 (+)	932.98	885.48	100	99.1	51.6
	FNQNWGLSGEVK (2+)	y8 (+)	689.84	875.46	100	81.4	39.5
		y7 (+)	689.84	689.38	100	81.4	39.5
		y5 (+)	689.84	519.28	100	81.4	39.5
Smlt0387	VGAGYNVEIAPSTDFVAR (2+)	y10 (+)	933.47	1076.57	100	99.2	51.7
		y9 (+)	933.47	963.49	100	99.2	51.7
		y8 (+)	933.47	892.45	100	99.2	51.7
	LNQNWGLNGELK (2+)	y8 (+)	693.36	916.49	100	81.7	39.7
		y7 (+)	693.36	730.41	100	81.7	39.7
		y5 (+)	693.36	560.30	100	81.7	39.7
Smlt2667	GVAPQDLR (2+)	y6 (+)	428.24	699.38	100	62.3	26.4
		y5 (+)	428.24	628.34	100	62.3	26.4
		y4 (+)	428.24	531.29	100	62.3	26.4
	IAYADSLSAPGYQLK (2+)	y10 (+)	798.92	1063.58	100	89.4	44.9
		y8 (+)	798.92	863.46	100	89.4	44.9
		y6 (+)	798.92	705.39	100	89.4	44.9
BSA	AEFVEVTK (2+)	y6 (+)	461.75	722.41	100	64.8	28.1
		y5 (+)	461.75	575.34	100	64.8	28.1
		y4 (+)	461.75	476.27	100	64.8	28.1
	QTALVELLK (2+)	y7 (+)	507.81	785.51	100	68.1	30.4
		y6 (+)	507.81	714.48	100	68.1	30.4
		y5 (+)	507.81	601.39	100	68.1	30.4

A4. Penicillin G tolerance plate assay

Optical density (OD600) of *S. maltophilia* cultures exposed to different concentrations of penicillin G (2 biological replicate analysis)

Penicillin G concentration (mg/ml)	Bio 1	Bio 2	Average	STD
0	0.593	0.613	0.603	0.014
0.01	0.636	0.628	0.632	0.006
0.05	0.569	0.517	0.543	0.037
0.1	0.523	0.512	0.518	0.008
0.5	0.601	0.558	0.580	0.030
1	0.615	0.564	0.590	0.036
5	0.024	0.014	0.019	0.007
10	0.004	-0.008	-0.002	0.008
50	0	-0.014	-0.007	0.010
100	0.054	0.002	0.028	0.037

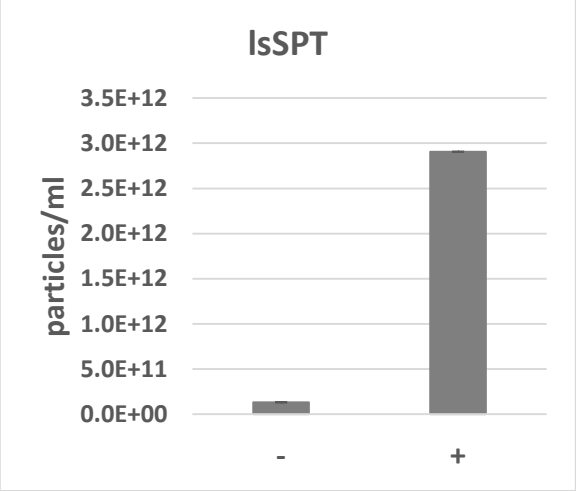


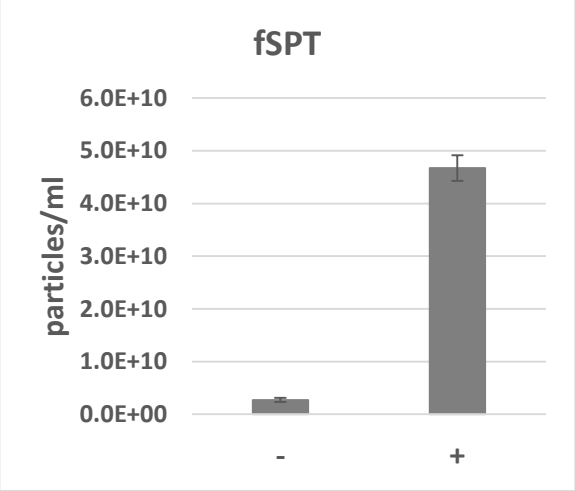
A5. Quantification of penicillin G induced OMVs

OMV quantification with light scattering based single particle tracking (lsSPT) and fluorescent single particle tracking (fSPT) (3 technical replicate analysis)

Method	Condition[a]	Particles/ml			Average	STD	Ratio
		Tech 1	Tech 2	Tech 3			+/-
lsSPT	**-**	1.354e11	1.290e11	1.276e11	1.306e11	2.079e7	22.23
	+	2.900e12	2.820e12	2.996e12	2.905e12	2.203e7	
fSPT	**-**	2.840e9	2.240e9	3.040e9	2.707e9	4.163e8	17.27
	+	4.610e10	4.940e10	4.470e10	4.673e10	2.413e9	

[a] control (-), penicillin G stimulated (+)





A6. Qualitative and quantitative comparison of the penicillin G- and imipenem-induced OMV proteome

Label-free quantitative analysis with Progenesis™

The 2D-LCMS^E data was loaded into the Progenesis™ LC-MS 4.0 software from Nonlinear Dynamics (Newcastle, UK), and the runs were aligned to a reference run (with the most common features with all runs). The detected features (charge > 1) were normalized, and peptides with different abundances in the penicillin G- and imipenem-OMV proteome data were further considered ($p \leq 0.01$). Identifications were further refined by excluding those with a mass error > 10 ppm and less than 2 peptide identification hits for the feature. Using the peptide abundances, proteins were quantified and subjected to similar statistic analysis ($p \leq 0.01$).

Protein identification list of the 2D-LCMS OMV proteome analysis with normalized abundances of differentially secreted OMV proteins (X: identified in that condition; NA: normalized abundance; * no significant difference in abundance; ** present in only one of the two conditions)

Entry	Gene names	Protein names	IMI	PEN	NA_{IMI}	NA_{PEN}	Fold
B2FIV0	groS groES Smlt4215	10 kDa chaperonin (GroES protein) (Protein Cpn10)	X	X	-	-	*
B2FNP5	rpsA Smlt2043	30S ribosomal protein S1	X	X	-	-	*
B2FN87	rpsO Smlt3386	30S ribosomal protein S15	X	X	3696.3	559.15	6.61
B2FIA9	rpsB Smlt1507	30S ribosomal protein S2	X		-	-	**
B2FQ51	rpsC Smlt0912	30S ribosomal protein S3	X	X	3060.16	1884.77	1.62
B2FJU4	rplM Smlt4302	50S ribosomal protein L13	X		-	-	**
B2FQ52	rplP Smlt0913	50S ribosomal protein L16	X		-	-	**
B2FQK9	rplQ Smlt0933	50S ribosomal protein L17	X	X	1102.42	466.89	2.36
B2FQ48	rplB Smlt0909	50S ribosomal protein L2	X	X	-	-	*
B2FN76	rplT Smlt3375	50S ribosomal protein L20	X	X	-	-	*
B2FTD1	rplU Smlt1278	50S ribosomal protein L21	X	X	-	-	*
B2FQ20	rplY ctc Smlt0876	50S ribosomal protein L25 (General stress protein CTC)	X	X	-	-	*
B2FQJ6	rplE Smlt0918	50S ribosomal protein L5	X	X	-	-	*
B2FQ37	rplL Smlt0897	50S ribosomal protein L7/L12	X	X	-	-	*
B2FKJ7	rplI Smlt3148	50S ribosomal protein L9	X	X	-	-	*
B2FIU9	groL groEL Smlt4214	60 kDa chaperonin (GroEL protein) (Protein Cpn60)	X	X	-	-	*
B2FU43	Smlt0084	Acid phosphatase (EC 3.1.3.2)	X	X	-	-	*
B2FT48	adk Smlt3886	Adenylate kinase (AK) (EC 2.7.4.3) (ATP-AMP transphosphorylase) (ATP:AMP phosphotransferase) (Adenylate monophosphate kinase)	X	X	-	-	*
B2FKK2	asnS Smlt3154	Asparagine--tRNA ligase (EC 6.1.1.22) (Asparaginyl-tRNA synthetase)	X		-	-	**
B2FHZ0	atpA Smlt4113	ATP synthase subunit alpha (EC 3.6.3.14) (ATP synthase F1 sector subunit alpha) (F-ATPase subunit alpha)	X		-	-	**
B2FHY8	atpD Smlt4111	ATP synthase subunit beta (EC 3.6.3.14) (ATP synthase F1 sector subunit beta) (F-ATPase subunit beta)	X	X	-	-	*
B2FUV6	hslU Smlt4075	ATP-dependent protease ATPase subunit HslU (Unfoldase HslU)	X		-	-	**
B2FRP5	Smlt3722	Beta-lactamase (EC 3.5.2.6)	X	X	-	-	*
B2FNP1	ftsA Smlt0759	Cell division protein ftsA	X		-	-	**
B2FTA6	minE Smlt1250	Cell division topological specificity factor	X		-	-	**
B2FMY5	dnaK Smlt1992	Chaperone protein DnaK (HSP70) (Heat shock 70 kDa protein) (Heat shock protein 70)	X	X	-	-	*

B2FLW2	htpG Smlt1809	Chaperone protein HtpG (Heat shock protein HtpG) (High temperature protein G)	X	X	-	-	*
B2FPG7	surA Smlt0820	Chaperone SurA (Peptidyl-prolyl cis-trans isomerase SurA) (Rotamase SurA)	X	X	11300	21400	1.9
B2FSI7	gltA Smlt3835	Citrate synthase (EC 2.3.3.16)	X	X	-	-	*
B2FI05	Smlt4128	Conserved hypothetical exported protein	X	X	-	-	*
B2FJC6	Smlt1634	Conserved hypothetical exported protein	X	X	-	-	*
B2FKV7	Smlt0483	Conserved hypothetical exported protein	X		-	-	**
B2FL62	Smlt1774	Conserved hypothetical exported protein	X	X	-	-	*
B2FLW5	Smlt1812	Conserved hypothetical exported protein	X	X	-	-	*
B2FMI3	Smlt4500	Conserved hypothetical exported protein	X	X	-	-	*
B2FMI4	Smlt4501	Conserved hypothetical exported protein		X	-	-	**
B2FN54	Smlt3351	Conserved hypothetical exported protein	X	X	-	-	*
B2FNH0	Smlt4628	Conserved hypothetical exported protein	X	X	-	-	*
B2FP55	Smlt4642	Conserved hypothetical exported protein	X	X	11300	25600	2.27
B2FPN6	Smlt2180	Conserved hypothetical exported protein		X	-	-	**
B2FQN8	Smlt0965	Conserved hypothetical exported protein	X		-	-	**
B2FQX4	Smlt2334	Conserved hypothetical exported protein	X	X	9848.42	16200	1.65
B2FQY5	Smlt2345	Conserved hypothetical exported protein	X	X	-	-	*
B2FR42	Smlt3680	Conserved hypothetical exported protein	X	X	-	-	*
B2FR48	Smlt3686	Conserved hypothetical exported protein	X	X	-	-	*
B2FRX3	Smlt1121	Conserved hypothetical exported protein	X	X	-	-	*
B2FRX4	Smlt1122	Conserved hypothetical exported protein	X	X	-	-	*
B2FRX9	Smlt1127	Conserved hypothetical exported protein	X	X	-	-	*
B2FRZ2	Smlt1140	Conserved hypothetical exported protein	X	X	655.2	958.01	1.46
B2FRZ3	Smlt1141	Conserved hypothetical exported protein		X	-	-	**
B2FSE9	Smlt3796	Conserved hypothetical exported protein	X	X	12900	7153.63	1.8
B2FTB2	Smlt1256	Conserved hypothetical exported protein	X	X	3182.75	1394.42	2.28
B2FUT1	Smlt4049	Conserved hypothetical exported protein		X	-	-	**
B2FN59	Smlt3358	Conserved hypothetical repetitive protein	X	X	-	-	*
B2FT86	Smlt0008	Conserved hypothetical TPR repeat family protein	X	X	16200	34100	2.11
B2FLD3	odhL Smlt3199	Dihydrolipoyl dehydrogenase (EC 1.8.1.4)	X		-	-	**
B2FQK8	rpoA Smlt0931	DNA-directed RNA polymerase subunit alpha (RNAP subunit alpha) (EC 2.7.7.6) (RNA polymerase subunit alpha) (Transcriptase subunit alpha)	X		-	-	**
B2FQ38	rpoB Smlt0898	DNA-directed RNA polymerase subunit beta (RNAP subunit beta) (EC 2.7.7.6) (RNA polymerase subunit beta) (Transcriptase subunit beta)	X		-	-	**
B2FQ39	rpoC Smlt0899	DNA-directed RNA polymerase subunit beta' (RNAP subunit beta') (EC 2.7.7.6) (RNA polymerase subunit beta') (Transcriptase subunit beta')	X		-	-	**
B2FQ42	fusA Smlt0903	Elongation factor G (EF-G)	X	X	3462.36	1785.86	1.94
B2FIA8	tsf Smlt1506	Elongation factor Ts (EF-Ts)	X	X	-	-	*
B2FQ31	tufB tuf tuf Smlt0890 Smlt0904	Elongation factor Tu (EF-Tu)	X	X	11200	18600	1.66
B2FK88	eno Smlt1715	Enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase) (2-phosphoglycerate dehydratase)	X	X	97.41	0	Infinity
B2FJU0	bfr Smlt4297	Ferroxidase (EC 1.16.3.1)	X	X	-	-	*
B2FQU5	fliC Smlt2304	Flagellin	X	X	9892.63	33600	3.39
B2FQU6	flaA Smlt2305	Flagellin	X	X	-	-	*
B2FQU7	Smlt2306	Flagellin	X	X	837.22	3129.84	3.74
B2FU50	opgD Smlt0091	Glucans biosynthesis protein D	X	X	1138.53	2400.54	2.11
B2FRW6	lptA Smlt1114	Lipopolysaccharide export system protein LptA	X	X	331.68	522.98	1.58
B2FQR4	lon Smlt0991	Lon protease (EC 3.4.21.53) (ATP-dependent protease La)	X		-	-	**
B2FPR6	lptE Smlt3484	LPS-assembly lipoprotein LptE	X	X	3802.32	6374.01	1.68
B2FPG8	lptD ostA Smlt0821	LPS-assembly protein LptD	X		-	-	**
B2FNJ0	smf-1 Smlt0706	Major fimbrial subunit SMF-1 (S. maltophilia fimbriae 1) (SMF-1)	X	X	-	-	*
B2FQL8	mdh Smlt0944	Malate dehydrogenase (EC 1.1.1.37)	X	X	1040.73	726.02	1.43
B2FNQ5	ndk Smlt2054	Nucleoside diphosphate kinase (NDK) (NDP kinase) (EC 2.7.4.6) (Nucleoside-2-P kinase)	X		-	-	**
B2FIA0	bamA Smlt1498	Outer membrane protein assembly factor BamA	X	X	12000	15900	1.33

B2FNR0	bamB Smlt2059	Outer membrane protein assembly factor BamB	X	X	13200	24000	1.82
B2FRR9	comL bamD Smlt3748	Outer membrane protein assembly factor BamD	X	X	4428.57	2111.78	2.1
B2FMX8	bamE Smlt1985	Outer membrane protein assembly factor BamE	X	X	-	-	*
B2FQY6	lolA Smlt2346	Outer-membrane lipoprotein carrier protein	X	X	-	-	*
B2FQ17	lolB Smlt0873	Outer-membrane lipoprotein LolB	X	X	2395.89	5059.7	2.11
B2FR43	mrcB Smlt3681	Penicillin-binding protein 1B (PBP-1b) (PBP1b) (Murein polymerase)	X	X	7857.59	3111.21	2.53
B2FLB8	Smlt3182	Peptidyl-prolyl cis-trans isomerase	X	X	-	-	*
B2FIF8	Smlt1559	Peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8)	X	X	2684.47	7155.85	2.67
B2FR55	Smlt0993	Peptidylprolyl isomerase (EC 5.2.1.8)	X		-	-	**
B2FN86	pnp Smlt3385	Polyribonucleotide nucleotidyltransferase (EC 2.7.7.8) (Polynucleotide phosphorylase) (PNPase)	X		-	-	**
B2FSQ8	mqo Smlt1234	Probable malate:quinone oxidoreductase (EC 1.1.5.4) (MQO) (Malate dehydrogenase [quinone])	X		-	-	**
B2FTS5	tolC Smlt3928	Protein CyaE	X	X	3975.49	2523.22	1.58
B2FMY4	grpE Smlt1991	Protein GrpE (HSP-70 cofactor)	X	X	-	-	*
B2FRM9	tolB Smlt3704	Protein TolB	X	X	32600	67300	2.06
B2FPB2	secA Smlt0764	Protein translocase subunit SecA	X	X	740.72	289.03	2.56
B2FHD7	secB Smlt0171	Protein-export protein SecB	X		-	-	**
B2FKN2	Smlt4330	Putative 4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27)	X		-	-	**
B2FP85	Smlt4673	Putative ABC transporter toluene tolerance exported protein	X	X	1887.04	3815.37	2.02
B2FT31	Smlt3869	Putative ACR family protein	X	X	1140.82	2871.11	2.52
B2FP33	Smlt3463	Putative alkaline phosphatase	X	X	5020.76	11300	2.24
B2FPY7	ahpC Smlt0841	Putative alkyl hydroperoxide reductase subunit c (EC 1.11.1.15)	X	X	-	-	*
B2FJX9	Smlt0418	Putative aminopeptidase	X		-	-	**
B2FL11	Smlt0541	Putative aminopeptidase	X	X	-	-	*
B2FSD8	Smlt3783	Putative aminopeptidase		X	-	-	**
B2FQE3	Smlt3574	Putative angiotensin-converting enzyme like peptidyl dipeptidase protein	X		-	-	**
B2FR62	Smlt1001	Putative autotransporter	X		-	-	**
B2FPV5	sphB Smlt3524	Putative autotransporter subtilisin-like protease	X	X	-	-	*
B2FT88	exbB1 Smlt0010	Putative biopolymer transport exbB protein	X	X	-	-	*
B2FT89	exbD1 Smlt0011	Putative biopolymer transport ExbD1 protein	X		-	-	**
B2FPM6	Smlt2170	Putative calcineurin phosphoesterase	X	X	-	-	*
B2FJY2	ctpA Smlt0421	Putative carboxy-terminal processing protease (EC 3.4.21.102)	X	X	4242.05	8272.46	1.95
B2FII6	Smlt2904	Putative decarboxylase		X	-	-	**
B2FK12	Smlt0451	Putative dehydrogenase	X	X	127.74	38.99	3.28
B2FUE8	dat Smlt1415	Putative diaminobutyrate--2-oxoglutarate aminotransferase (EC 2.6.1.76)	X	X	533.53	125.1	4.26
B2FP98	Smlt4686	Putative dipeptidase		X	-	-	**
B2FSF8	Smlt3806	Putative endonuclease P1 (EC 3.1.30.1)	X	X	-	-	*
B2FP18	pepO Smlt3447	Putative endopeptidase O (EC 3.4.24.-)	X		-	-	**
B2FMI6	Smlt4503	Putative exported dipeptidyl peptidase IV	X	X	5220.39	2101.49	2.48
B2FP20	Smlt3450	Putative exported endopeptidase	X	X	-	-	*
B2FI03	Smlt4126	Putative exported lipoprotein	X		-	-	**
B2FNA4	Smlt4560	Putative exported lipoprotein		X	-	-	**
B2FKH7	Smlt3128	Putative exported oligopeptidase		X	-	-	**
B2FHW1	Smlt4084	Putative exported oligoPEPTIDASE (EC 3.4.-.-)		X	-	-	**
B2FTA3	Smlt1246	Putative exported peptidase (EC 3.4.14.-)	X	X	5114	10200	2
B2FUT3	rlpA Smlt4051	Putative exported rare lipoprotein A	X	X	2113.46	1023.15	2.07
B2FUS7	Smlt4045	Putative exported tail-specific protease (EC 3.4.21.102)	X	X	-	-	*
B2FJY4	Smlt0423	Putative fatty acid transport system, membrane protein	X	X	-	-	*
B2FR50	Smlt3688	Putative ferritin DPS-family DNA binding protein	X	X	4502.44	619.55	7.27
B2FNJ3	Smlt0709	Putative fimbria adhesin protein	X	X	8706.82	16200	1.86
B2FNQ7	Smlt2056	Putative fimbrial biogenesis protein	X	X	-	-	*
B2FIR8	Smlt4182	Putative fimbrial protein (Pilin)	X		-	-	**
B2FST5	Smlt2569	Putative glucan 1,4-beta-glucosidase	X	X	-	-	*
B2FP87	Smlt4675	Putative intercellular spreading VacJ lipoprotein	X	X	-	-	*
B2FRZ9	Smlt1148	Putative iron transport receptor protein	X	X	-	-	*

B2FHT9	Smlt2858	Putative iron transporter	X		-	-	**
B2FJR8	icd Smlt4273	Putative isocitrate dehydrogenase [NADP] (EC 1.1.1.42)	X		-	-	**
B2FQQ5	Smlt0982	Putative isocitrate/isopropylmalate dehydrogenase	X	X	-	-	*
B2FP30	Smlt3460	Putative lipoprotein	X	X	796.29	234.89	3.39
B2FRR0	Smlt3739	Putative lipoprotein		X	-	-	**
B2FUS6	Smlt4044	Putative lipoprotein	X		-	-	**
B2FU57	hel Smlt0098	Putative lipoprotein E (Outer membrane protein p4)	X		-	-	**
B2FNM5	Smlt0742	Putative LppC family lipoprotein	X	X	-	-	*
B2FP06	mltD Smlt3434	Putative membrane-bound lytic murein transglycosylase d (EC 3.2.1.-)	X	X	-	-	*
B2FTM1	Smlt2667	Putative metallo-beta-lactamase l1 (Beta-lactamase type ii) (Ec 3.5.2.6) (Penicillinase) (EC 3.5.2.6)	X	X	-	-	*
B2FRQ3	Smlt3731	Putative MltA scaffolding protein	X	X	-	-	*
B2FH96	Smlt0129	Putative modulator of DNA gyrase	X	X	689.48	1511.75	2.19
B2FUT4	mltB Smlt4052	Putative murein hydrolase (EC 3.2.1.-)	X	X	-	-	*
B2FHC0	Smlt0154	Putative N-acetylmuramoyl-L-alanine amidase	X	X	-	-	*
B2FN37	Smlt3330	Putative N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)		X	-	-	**
B2FJB1	Smlt1619	Putative oar family adhesion protein	X	X	-	-	*
B2FLE9	Smlt3215	Putative outer membrane antigen lipoprotein	X	X	-	-	*
B2FLE4	Smlt3210	Putative outer membrane antigen protein	X	X	6869.42	4204.4	1.63
B2FLY6	smeX Smlt1833	Putative outer membrane efflux protein	X	X	-	-	*
B2FSC8	Smlt3773	Putative outer membrane esterase	X	X	-	-	*
B2FLX9	Smlt1826	Putative outer membrane lipoprotein	X	X	20700	35300	1.71
B2FPV8	Smlt3527	Putative outer membrane lipoprotein	X	X	847.02	2569.56	3.03
B2FR78	Smlt1018	Putative outer membrane lipoprotein	X	X	-	-	*
B2FI00	Smlt4123	Putative outer membrane Omp family protein	X	X	-	-	*
B2FSF7	Smlt3805	Putative outer membrane Omp family protein	X	X	-	-	*
B2FHW3	Smlt4086	Putative outer membrane protein		X	-	-	**
B2FLU3	Smlt0613	Putative outer membrane protein	X	X	156.25	569.24	3.64
B2FLU4	Smlt0614	Putative outer membrane protein	X	X	-	-	*
B2FQM8	Smlt0955	Putative outer membrane protein	X	X	-	-	*
B2FSS7	rpfN Smlt2559	Putative outer membrane regulator of pathogenicity factors protein	X	X	-	-	*
B2FNJ2	mrkC Smlt0708	Putative outer membrane usher protein mrkc	X	X	-	-	*
B2FU40	Smlt0080	Putative patatin-like phospholipase	X	X	-	-	*
B2FUT2	dacC Smlt4050	Putative penicillin-binding protein (EC 3.4.16.4)	X	X	-	-	*
B2FHG8	Smlt0203	Putative peptidase	X		-	-	**
B2FJ20	Smlt0348	Putative peptidase	X	X	5842.03	12000	2.05
B2FT85	Smlt0007	Putative peptidase	X	X	3769.87	5556.8	1.47
B2FUU5	Smlt4064	Putative PEPTIDASE	X	X	1765.56	578.87	3.05
B2FH98	Smlt0131	Putative peptidase/modulator of DNA gyrase		X	-	-	**
B2FRM8	Smlt3703	Putative peptidoglycan-associated lipoprotein	X	X	-	-	*
B2FLG2	Smlt3229	Putative peptidyl dipeptidase/oligopeptidase	X	X	5726.61	12100	2.11
B2FI43	dcp Smlt0223	Putative peptidyl-dipeptidase Dcp (Dipeptidyl carboxypeptidase) (EC 3.4.15.5)	X	X	7953.54	2939.36	2.71
B2FHM5	Smlt1483	Putative peptidyl-prolyl cis-trans isomerase		X	-	-	**
B2FTU3	Smlt3950	Putative phosphate selective porin	X	X	1357.55	2424.36	1.79
B2FHJ5	Smlt1449	Putative phosphodiesterase-nucleotide pyrophosphatase	X	X	-	-	*
B2FNJ1	Smlt0707	Putative pili chaperone protein	X	X	-	-	*
B2FTT7	oprP Smlt3943	Putative porin P (Outer membrane protein d1)	X	X	12600	25000	1.98
B2FJ52	Smlt0381	Putative protease	X	X	12700	24200	1.91
B2FUA1	qoxB Smlt1361	Putative quinol oxidase subunit 1	X		-	-	**
B2FUU0	mreB Smlt4059	Putative rod shape-determining protein	X		-	-	**
B2FRW3	Smlt1111	Putative sigma(54) modulation protein	X	X	-	-	*
B2FK97	Smlt1724	Putative subfamily M23B unassigned peptidase	X	X	-	-	*
B2FQC5	Smlt3553	Putative subfamily S1C unassigned peptidase	X	X	23800	57400	2.41
B2FIM6	dsbG Smlt2946	Putative thiol:disulfide interchange protein		X	-	-	**
B2FTY8	Smlt3994	Putative thiol:disulfide interchange protein	X	X	-	-	*
B2FPV6	Smlt3525	Putative thiolase		X	-	-	**
B2FQ11	Smlt0866	Putative thioredoxin electron transport related protein	X	X	-	-	*
B2FPR9	Smlt3487	Putative thioredoxin protein		X	-	-	**

B2FRN0	tolA Smlt3705	Putative TolA transmembrane protein	X		-	-	**
B2FRN2	tolQ Smlt3707	Putative TolQ transport transmembrane protein	X		-	-	**
B2FRN1	tolR Smlt3706	Putative TolR-related protein	X	X	2415.3	1479.65	1.63
B2FLT5	Smlt0602	Putative TonB dependent receptor		X	-	-	**
B2FN47	Smlt3340	Putative TonB dependent receptor	X	X	-	-	*
B2FP16	Smlt3444	Putative TonB dependent receptor	X	X	-	-	*
B2FR08	Smlt3645	Putative TonB dependent receptor	X	X	10700	21900	2.05
B2FRP8	Smlt3725	Putative TonB dependent receptor	X	X	-	-	*
B2FRR1	Smlt3740	Putative TonB dependent receptor	X	X	2037.95	5091.01	2.5
B2FST2	Smlt2566	Putative TonB dependent receptor	X	X	4419.08	8846.28	2
B2FT66	Smlt3905	Putative TonB dependent receptor	X	X	-	-	*
B2FJ30	Smlt0359	Putative TonB dependent receptor protein	X	X	24900	11400	2.18
B2FKT0	Smlt4387	Putative TonB dependent receptor protein	X		-	-	**
B2FM99	Smlt3254	Putative TonB dependent receptor protein	X	X	-	-	*
B2FP17	Smlt3446	Putative TonB dependent receptor protein	X	X	-	-	*
B2FU42	Smlt0083	Putative TonB dependent receptor protein	X	X	15400	23700	1.54
B2FHH2	Smlt1426	Putative TonB dependent siderophore receptor	X		-	-	**
B2FKE5	Smlt3093	Putative TonB domain protein	X	X	97.21	1.43	68.11
B2FHR9	Smlt2835	Putative TonB-dependent outer membrane protein		X	-	-	**
B2FPN5	Smlt2179	Putative TonB-dependent outer membrane receptor protein	X	X	-	-	*
B2FUR1	Smlt4026	Putative TonB-dependent outer membrane receptor protein	X	X	-	-	*
B2FRC4	Smlt1067	Putative TonB-dependent receptor	X	X	583.18	1196.18	2.05
B2FRM7	Smlt3702	Putative TPR repeat exported protein	X	X	7715.31	15900	2.06
B2FNV9	Smlt2112	Putative transcriptional regulator-TetR family		X	-	-	**
B2FUP4	Smlt4007	Putative transglycosylase	X	X	-	-	*
B2FP62	Smlt4650	Putative transglycosylase protein	X	X	-	-	*
B2FL08	Smlt0538	Putative transmembrane anchor protein	X	X	-	-	*
B2FHD1	Smlt0165	Putative transmembrane HemY porphyrin biosynthesis protein	X		-	-	**
B2FHE8	Smlt0182	Putative transmembrane protein	X	X	-	-	*
B2FN01	Smlt2010	Putative transmembrane protein	X	X	-	-	*
B2FQ16	Smlt0872	Putative transmembrane protein	X	X	2554.26	4920.61	1.93
B2FIV3	Smlt4218	Putative transmembrane Thiol:disulfide Interchange Protein	X		-	-	**
B2FI94	Smlt0278	Putative two component regulator sensor histidine kinase transmembrane transcriptional regulatory protein	X		-	-	**
B2FLR8	Smlt0585	Putative vitamin B12 receptor protein	X	X	-	-	*
B2FQZ2	Smlt3629	Putative YciI family periplasmic protein		X	-	-	**
B2FJ75	potF Smlt1581	Putrescine-binding periplasmic protein	X	X	1270.62	526.71	2.41
B2FM92	rnE rne Smlt3247	Ribonuclease E (RNase E) (EC 3.1.26.12)	X		-	-	**
B2FPY1	lepB Smlt3551	Signal peptidase I (EC 3.4.21.89)	X		-	-	**
B2FTA7	minD Smlt1251	Site-determining protein	X	X	-	-	*
B2FRS2	sucD Smlt3752	Succinyl-CoA ligase [ADP-forming] subunit alpha (EC 6.2.1.5)	X	X	-	-	*
B2FLH1	sodA Smlt3238	Superoxide dismutase (EC 1.15.1.1)		X	-	-	**
B2FHC6	sodC1 Smlt0160	Superoxide dismutase [Cu-Zn] (EC 1.15.1.1)	X	X	-	-	*
B2FHC7	sodC2 Smlt0161	Superoxide dismutase [Cu-Zn] (EC 1.15.1.1)	X		-	-	**
B2FTY7	dsbA Smlt3993	Thiol:disulfide interchange protein	X	X	171.74	30.82	5.57
B2FQ33	nusG Smlt0892	Transcription termination/antitermination protein NusG	X		-	-	**
B2FQR1	tig Smlt0988	Trigger factor (TF) (EC 5.2.1.8) (PPIase)	X		-	-	**
B2FHB5	Smlt0149	Uncharacterized protein	X	X	8722.45	5246.92	1.66
B2FHE7	Smlt0181	Uncharacterized protein	X	X	1739.79	2790.15	1.6
B2FHF0	Smlt0184	Uncharacterized protein	X	X	-	-	*
B2FHG9	Smlt0204	Uncharacterized protein	X	X	7445.79	14000	1.88
B2FHL9	Smlt1474	Uncharacterized protein	X		-	-	**
B2FHN9	Smlt2799	Uncharacterized protein	X		-	-	**
B2FHZ6	Smlt4119	Uncharacterized protein	X	X	-	-	*
B2FII7	Smlt2905	Uncharacterized protein	X	X	-	-	*
B2FIM5	Smlt2944	Uncharacterized protein	X	X	-	-	*

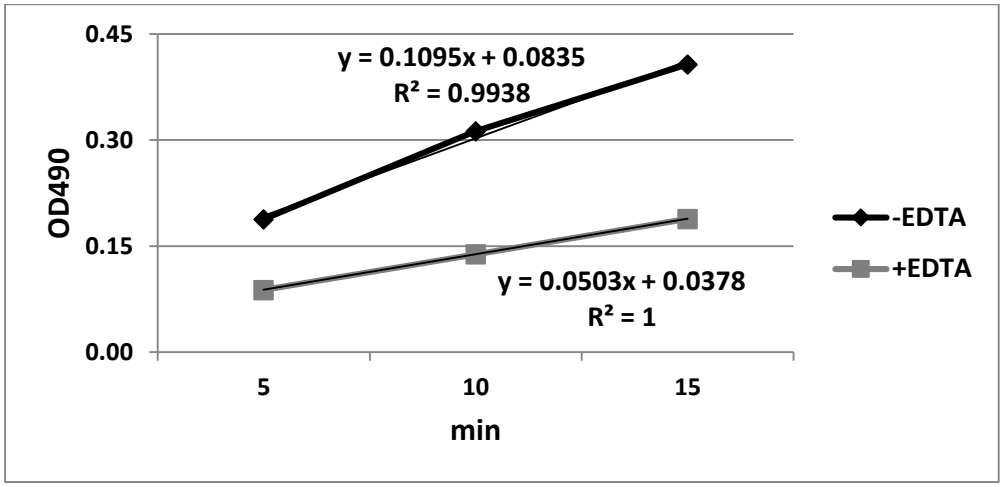
B2FJR9	Smlt4275	Uncharacterized protein	X		-	-	**
B2FJV0	Smlt0387	Uncharacterized protein	X	X	-	-	*
B2FJV9	Smlt0397	Uncharacterized protein	X		-	-	**
B2FL09	Smlt0539	Uncharacterized protein	X	X	1622.76	707.26	2.29
B2FLG5	Smlt3232	Uncharacterized protein	X	X	87.66	0.34	258.54
B2FME4	Smlt3304	Uncharacterized protein	X	X	734.91	2366.12	3.22
B2FND4	Smlt4590	Uncharacterized protein	X	X	123.11	551.26	4.48
B2FND7	Smlt4593	Uncharacterized protein	X	X	-	-	*
B2FNK1	Smlt0717	Uncharacterized protein	X		-	-	**
B2FNK9	Smlt0725	Uncharacterized protein	X	X	637.96	3960.14	6.21
B2FPC4	Smlt0777	Uncharacterized protein		X	-	-	**
B2FPU4	Smlt3512	Uncharacterized protein		X	-	-	**
B2FQ57	Smlt2204	Uncharacterized protein	X	X	-	-	*
B2FQN1	Smlt0958	Uncharacterized protein	X	X	1436.51	4755.13	3.31
B2FQN3	Smlt0960	Uncharacterized protein	X	X	-	-	*
B2FQN5	Smlt0962	Uncharacterized protein	X	X	9007.29	24900	2.76
B2FT51	Smlt3889	Uncharacterized protein		X	-	-	**
B2FTS4	pcm Smlt3927	Uncharacterized protein	X		-	-	**
B2FUD6	Smlt1403	Uncharacterized protein	X	X	-	-	*
B2FUU4	Smlt4063	Uncharacterized protein	X		-	-	**

A7. Nitrocefin assay on intact OMVs

UV-VIS spectroscopy detection of hydrolyzed nitrocefin at OD490 during incubation with intact penicillin G induced OMVs, with and without the addition of 50 mM EDTA (2 biological replicate analysis)

	Bio 1		Bio 2		Average		STD	
min	**-EDTA**	**+EDTA**	**-EDTA**	**+EDTA**	**-EDTA**	**+EDTA**	**-EDTA**	**+EDTA**
5	0.201	0.096	0.175	0.080	0.188	0.088	0.018	0.011
10	0.334	0.154	0.291	0.123	0.313	0.139	0.030	0.022
15	0.431	0.212	0.383	0.165	0.407	0.189	0.034	0.033
20	0.481	0.250	0.438	0.203	0.460	0.227	0.030	0.033
25	0.514	0.291	0.483	0.230	0.499	0.261	0.022	0.043
30	0.537	0.331	0.514	0.265	0.526	0.298	0.016	0.047
35	0.544	0.354	0.526	0.286	0.535	0.320	0.013	0.048
40	0.549	0.379	0.534	0.311	0.542	0.345	0.011	0.048
45	0.553	0.404	0.542	0.331	0.548	0.368	0.008	0.052
50	0.552	0.423	0.545	0.352	0.549	0.388	0.005	0.050
55	0.554	0.441	0.549	0.371	0.552	0.406	0.004	0.049
60	0.558	0.455	0.548	0.387	0.553	0.421	0.007	0.048

Rate of hydrolysis derived from linear part of the curve

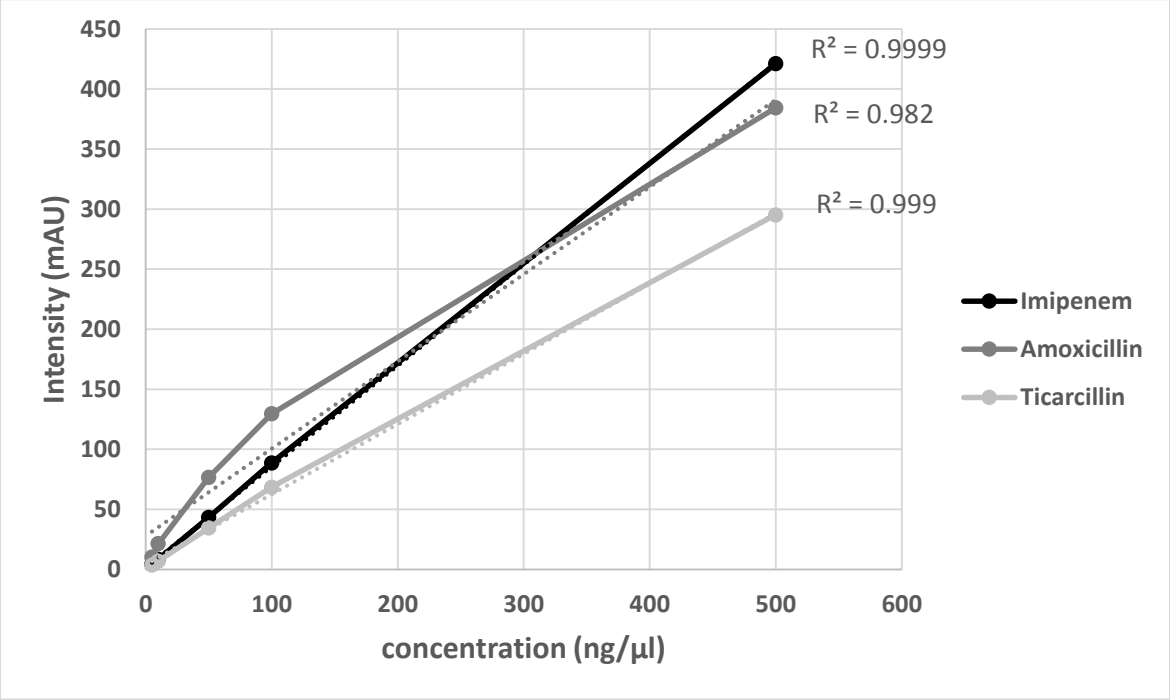


	ΔOD(t_{15}-t_5)	c = A (ΔOD)/ε (M)	c (µmol/ml)	c (µg/ml)	rate (µg/ml.min)
-EDTA	0.219	1.068E-05	0.011	5.710	**0.571**
+EDTA	0.101	4.902E-06	0.005	2.620	**0.262**

ε (hydrolyzed nitrocefin) ($M^{-1}.cm^{-1}$)	20500
MW (hydrolyzed nitrocefin) (µg/µmol)	534.5

A8. RP-HPLC linear dynamic range of imipenem, amoxicillin and ticarcillin

Concentration (ng/µl)	Imipenem Retention time (min)	Intensity (mAU)	Amoxicillin Retention time (min)	Intensity (mAU)	Ticarcillin Retention time (min)	Intensity (mAU)
5	2.42	4.49	3.42	10.35	3.66	3.74
10	2.41	8.50	3.40	21.43	3.65	6.95
50	2.40	43.32	3.39	76.57	3.65	34.45
100	2.40	88.75	3.42	129.58	3.64	68.47
500	2.43	421.12	3.41	384.27	3.62	295.13



A9. OMV concentration and size determination

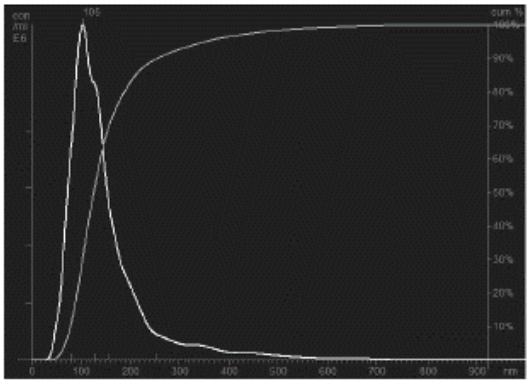
Raw data from OMV concentration and size determination by light scattering based single particle tracking (NanoSight LM10-HS)

- **'ctrl 1' > control sample, biological replicate 1**
- **'ctrl 3' > control sample, biological replicate 2**
- **'imi 1' > imipenem sample, biological replicate 1**
- **'imi 3' > imipenem sample, biological replicate 2**
- **'cip 1' > ciprofloxacin sample, biological replicate 1**
- **'cip 3' > ciprofloxacin sample, biological replicate 2**

NANOSIGHT

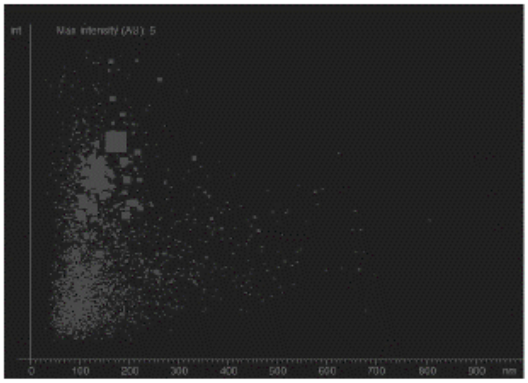
Nanoparticle Tracking Analysis (NTA) Version 2.3 Build 0034

ANALYSIS REPORT

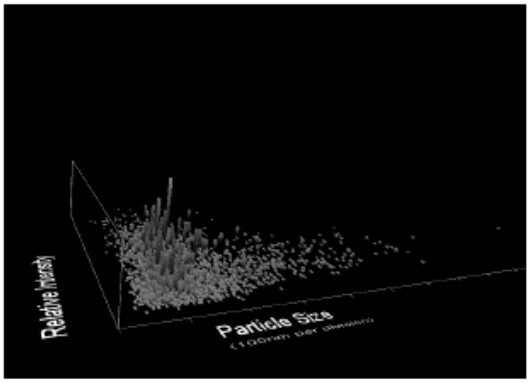


Particle Size / Concentration

Sample Video Frame



Particle Size / Relative Intensity



Particle Size / Relative Intensity 3D plot

Operator:	
Sample:	
Date/Time of Capture:	
Video File:	"ctrl 1 1 to 50.avi"
Analysis No:	001
Date/Time of Report:	12/08/2015 15:40:03
Dispersant/Diluent:	
Concentration:	
Pre-treatment:	
Remarks:	None

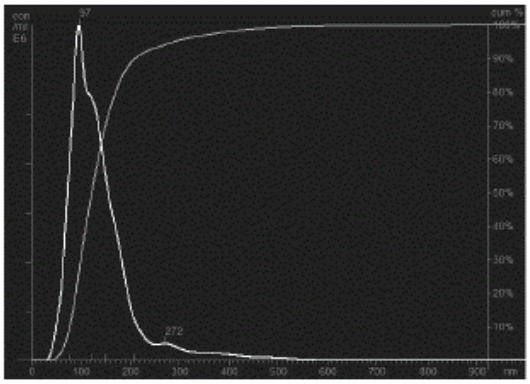
RESULTS:	
Size Distribution:	Mean: 155 nm, Mode: 105 nm, SD: 99 nm
Cumulative Data (nm):	D10: 80, D50: 128, D90: 255, D70: 159
User Lines:	0 nm, 0 nm
Total Concentration:	50.93 particles / frame, 6.10E8 particles / ml
Selected Concentration:	0.00 particles / frame, 0.00E8 particles / ml
Fitted Curve :	Mean: 0 nm, SD: 0
Completed Tracks:	2903
Drift Velocity:	70 nm/s

ANALYSIS SETTINGS:	
Frames Processed:	1499 of 1499
Frames per Second:	25.00
Calibration:	186 nm/pixel
Blur:	Auto
Detection Threshold:	10 Multi
Min Track Length:	Auto
Min Expected Size:	Auto
Temperature:	28.30 °C
Viscosity:	0.83 cP

NANOSIGHT

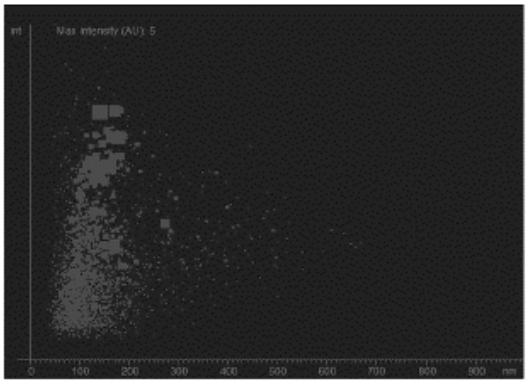
Nanoparticle Tracking Analysis (NTA) Version 2.3 Build 0034

ANALYSIS REPORT

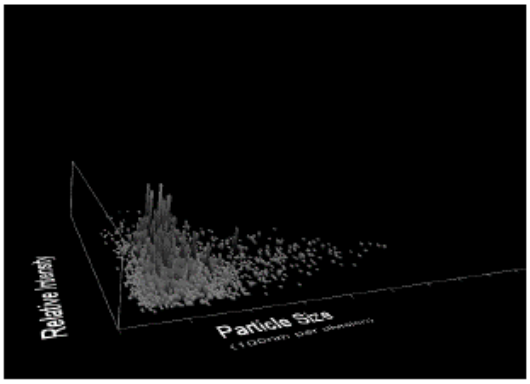


Particle Size / Concentration

Sample Video Frame



Particle Size / Relative Intensity



Particle Size / Relative Intensity 3D plot

Operator:	
Sample:	
Date/Time of Capture:	
Video File:	'ctrl 3 1 to 50.avi'
Analysis No:	001
Date/Time of Report:	12/08/2015 15:43:56
Dispersant/Diluent:	
Concentration:	
Pre-treatment:	
Remarks:	None

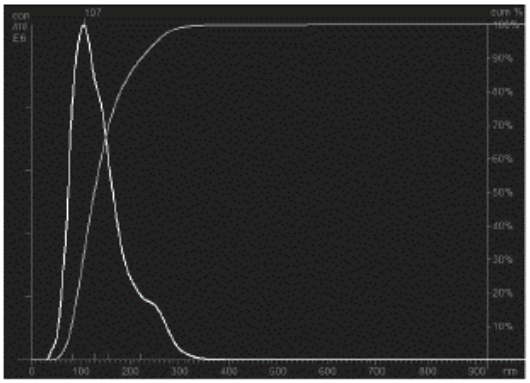
RESULTS:	
Size Distribution:	Mean: 142 nm, Mode: 97 nm, SD: 75 nm
Cumulative Data (nm):	D10: 79, D50: 124, D90: 210, D70: 151
User Lines:	0 nm, 0 nm
Total Concentration:	54.50 particles / frame, 6.48E8 particles / ml
Selected Concentration:	0.00 particles / frame, 0.00E8 particles / ml
Fitted Curve :	Mean: 0 nm, SD: 0
Completed Tracks:	2993
Drift Velocity:	79 nm/s

ANALYSIS SETTINGS:	
Frames Processed:	1499 of 1499
Frames per Second:	24.99
Calibration:	186 nm/pixel
Blur:	Auto
Detection Threshold:	10 Multi
Min Track Length:	Auto
Min Expected Size:	Auto
Temperature:	28.50 °C
Viscosity:	0.82 cP

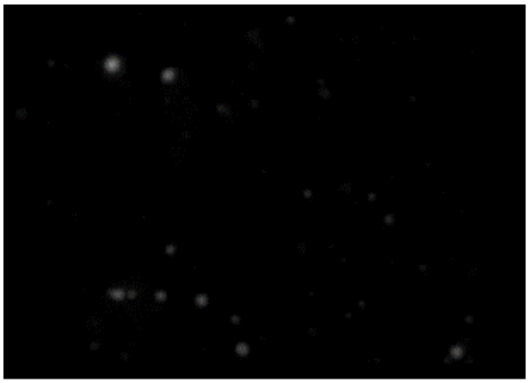
NANOSIGHT

Nanoparticle Tracking Analysis (NTA) Version 2.3 Build 0034

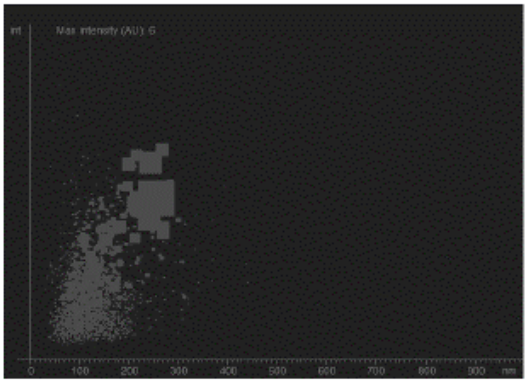
ANALYSIS REPORT



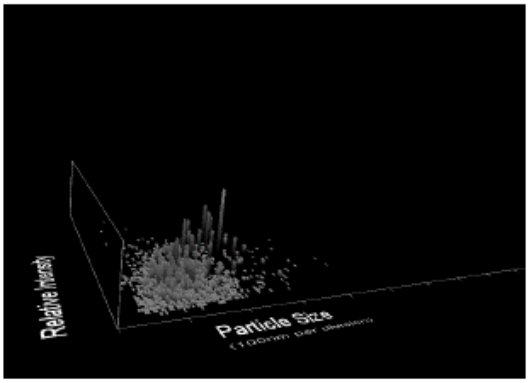
Particle Size / Concentration



Sample Video Frame



Particle Size / Relative Intensity



Particle Size / Relative Intensity 3D plot

Operator:	
Sample:	
Date/Time of Capture:	
Video File:	'imi 1 1to5000.avi'
Analysis No:	001
Date/Time of Report:	12/08/2015 14:35:15
Dispersant/Diluent:	
Concentration:	
Pre-treatment:	
Remarks:	None

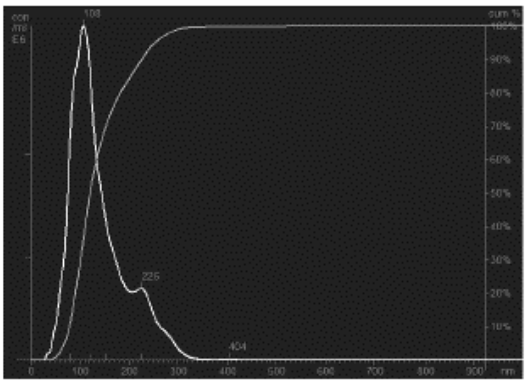
RESULTS:	
Size Distribution:	Mean: 142 nm, Mode: 107 nm, SD: 54 nm
Cumulative Data (nm):	D10: 83, D50: 129, D90: 224, D70: 159
User Lines:	0 nm, 0 nm
Total Concentration:	48.12 particles / frame, 5.72E8 particles / ml
Selected Concentration:	0.00 particles / frame, 0.00E8 particles / ml
Fitted Curve :	Mean: 0 nm, SD: 0
Completed Tracks:	2430
Drift Velocity:	220 nm/s

ANALYSIS SETTINGS:	
Frames Processed:	1499 of 1499
Frames per Second:	24.99
Calibration:	186 nm/pixel
Blur:	Auto
Detection Threshold:	10 Multi
Min Track Length:	Auto
Min Expected Size:	Auto
Temperature:	27.60 °C
Viscosity:	0.84 cP

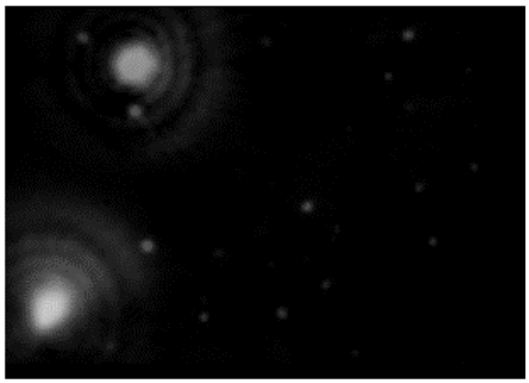
NANOSIGHT

Nanoparticle Tracking Analysis (NTA) Version 2.3 Build 0034

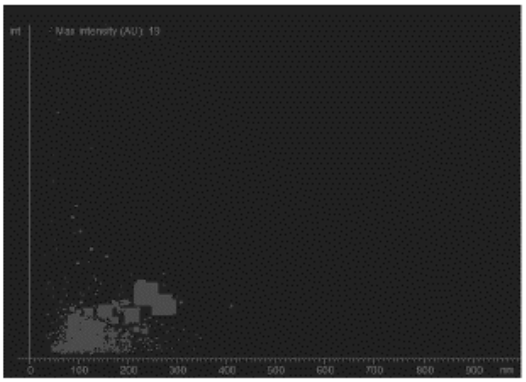
ANALYSIS REPORT



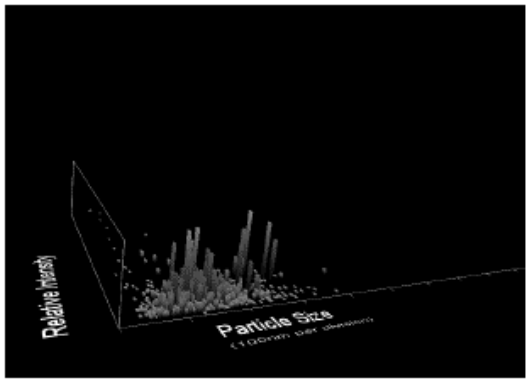
Particle Size / Concentration



Sample Video Frame



Particle Size / Relative Intensity



Particle Size / Relative Intensity 3D plot

Operator:	
Sample:	
Date/Time of Capture:	
Video File:	'imi 3 1to5000.avi'
Analysis No:	001
Date/Time of Report:	12/08/2015 14:42:59
Dispersant/Diluent:	
Concentration:	
Pre-treatment:	
Remarks:	None

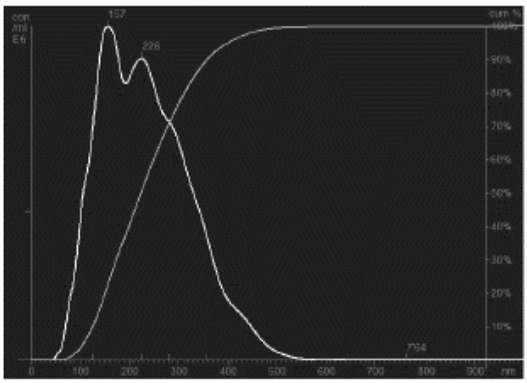
RESULTS:	
Size Distribution:	Mean: 139 nm, Mode: 108 nm, SD: 57 nm
Cumulative Data (nm):	D10: 80, D50: 122, D90: 227, D70: 154
User Lines:	0 nm, 0 nm
Total Concentration:	25.15 particles / frame, 3.07E8 particles / ml
Selected Concentration:	0.00 particles / frame, 0.00E8 particles / ml
Fitted Curve :	Mean: 0 nm, SD: 0
Completed Tracks:	1244
Drift Velocity:	113 nm/s

ANALYSIS SETTINGS:	
Frames Processed:	1499 of 1499
Frames per Second:	25.00
Calibration:	186 nm/pixel
Blur:	Auto
Detection Threshold:	10 Multi
Min Track Length:	Auto
Min Expected Size:	Auto
Temperature:	27.70 °C
Viscosity:	0.84 cP

NANOSIGHT

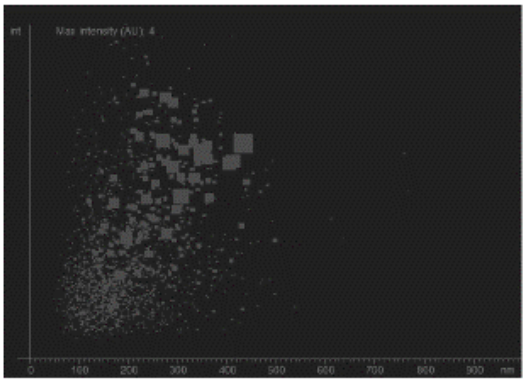
Nanoparticle Tracking Analysis (NTA) Version 2.3 Build 0034

ANALYSIS REPORT

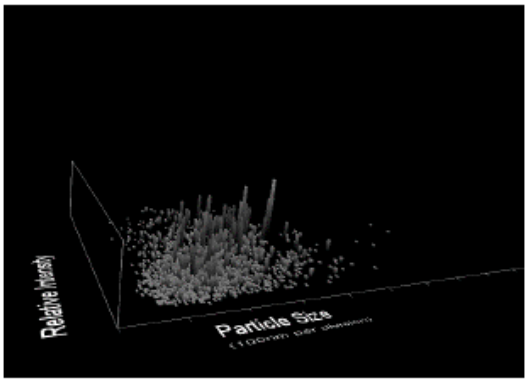


Particle Size / Concentration

Sample Video Frame



Particle Size / Relative Intensity



Particle Size / Relative Intensity 3D plot

Operator:	
Sample:	
Date/Time of Capture:	
Video File:	'CIP 1 1to1000.avi'
Analysis No:	001
Date/Time of Report:	12/08/2015 15:26:00
Dispersant/Diluent:	
Concentration:	
Pre-treatment:	
Remarks:	None

RESULTS:

Size Distribution:	Mean: 236 nm, Mode: 157 nm, SD: 91 nm
Cumulative Data (nm):	D10: 126, D50: 225, D90: 359, D70: 280
User Lines:	0 nm, 0 nm
Total Concentration:	42.46 particles / frame, 5.03E8 particles / ml
Selected Concentration:	0.00 particles / frame, 0.00E8 particles / ml
Fitted Curve :	Mean: 0 nm, SD: 0
Completed Tracks:	1763
Drift Velocity:	40 nm/s

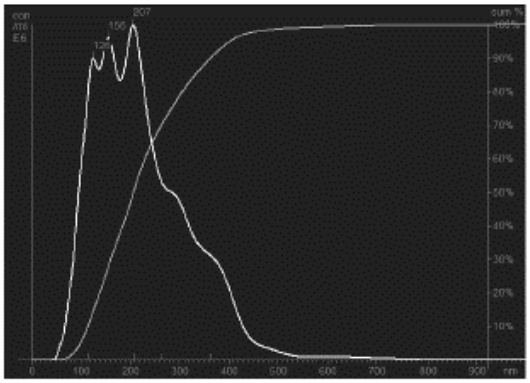
ANALYSIS SETTINGS:

Frames Processed:	1499 of 1499
Frames per Second:	24.99
Calibration:	186 nm/pixel
Blur:	Auto
Detection Threshold:	10 Multi
Min Track Length:	Auto
Min Expected Size:	Auto
Temperature:	28.30 °C
Viscosity:	0.83 cP

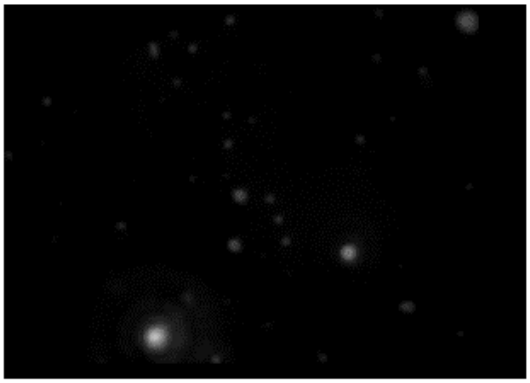
NANOSIGHT

Nanoparticle Tracking Analysis (NTA) Version 2.3 Build 0034

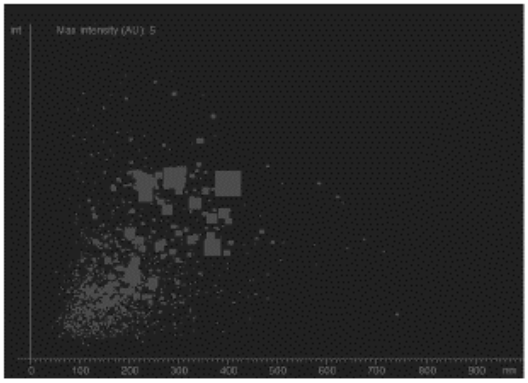
ANALYSIS REPORT



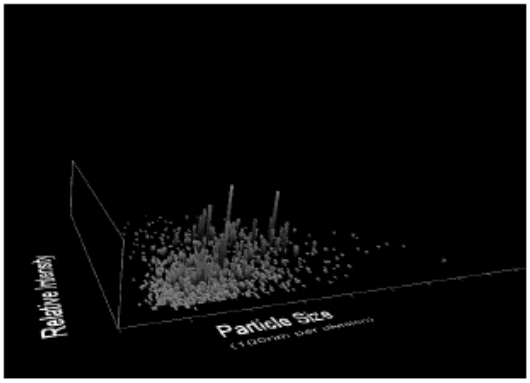
Particle Size / Concentration



Sample Video Frame



Particle Size / Relative Intensity



Particle Size / Relative Intensity 3D plot

Operator:	
Sample:	
Date/Time of Capture:	
Video File:	'CIP 3 1to1000.avi'
Analysis No:	001
Date/Time of Report:	12/08/2015 15:29:56
Dispersant/Diluent:	
Concentration:	
Pre-treatment:	
Remarks:	None

RESULTS:	
Size Distribution:	Mean: 226 nm, Mode: 207 nm, SD: 98 nm
Cumulative Data (nm):	D10: 115, D50: 208, D90: 363, D70: 264
User Lines:	0 nm, 0 nm
Total Concentration:	33.57 particles / frame, 3.95E8 particles / ml
Selected Concentration:	0.00 particles / frame, 0.00E8 particles / ml
Fitted Curve :	Mean: 0 nm, SD: 0
Completed Tracks:	1416
Drift Velocity:	65 nm/s

ANALYSIS SETTINGS:	
Frames Processed:	1499 of 1499
Frames per Second:	25.00
Calibration:	186 nm/pixel
Blur:	Auto
Detection Threshold:	10 Multi
Min Track Length:	Auto
Min Expected Size:	Auto
Temperature:	28.30 °C
Viscosity:	0.83 cP

A10. Qualitative comparison of the penicillin G- and imipenem-induced OMV proteome

Protein identification list of the 2D-LCMS OMV proteome analysis (identified in minimum 2/3 biological replicate runs) (X: identified in that condition)

Entry	Protein names	Gene names	IMI	CIP
B2FIV0	10 kDa chaperonin (GroES protein) (Protein Cpn10)	groS groES Smlt4215	X	
B2FNP5	30S ribosomal protein S1	rpsA Smlt2043		X
B2FQK6	30S ribosomal protein S11	rpsK Smlt0929		X
B2FQK5	30S ribosomal protein S13	rpsM Smlt0928		X
B2FIA9	30S ribosomal protein S2	rpsB Smlt1507		X
B2FQ51	30S ribosomal protein S3	rpsC Smlt0912	X	X
B2FQK7	30S ribosomal protein S4	rpsD Smlt0930		X
B2FQK1	30S ribosomal protein S5	rpsE Smlt0923		X
B2FQ41	30S ribosomal protein S7	rpsG Smlt0902		X
B2FQ35	50S ribosomal protein L1	rplA Smlt0895		X
B2FQ36	50S ribosomal protein L10	rplJ Smlt0896		X
B2FQ34	50S ribosomal protein L11	rplK Smlt0894		X
B2FJU4	50S ribosomal protein L13	rplM Smlt4302		X
B2FQJ4	50S ribosomal protein L14	rplN Smlt0916		X
B2FQK3	50S ribosomal protein L15	rplO Smlt0925		X
B2FQ52	50S ribosomal protein L16	rplP Smlt0913		X
B2FQK9	50S ribosomal protein L17	rplQ Smlt0933		X
B2FQK0	50S ribosomal protein L18	rplR Smlt0922		X
B2FUB6	50S ribosomal protein L19	rplS Smlt1377		X
B2FQ48	50S ribosomal protein L2	rplB Smlt0909	X	X
B2FQ47	50S ribosomal protein L23	rplW Smlt0908		X
B2FQ20	50S ribosomal protein L25 (General stress protein CTC)	rplY ctc Smlt0876		X
B2FTD2	50S ribosomal protein L27	rpmA Smlt1279		X
B2FQ45	50S ribosomal protein L3	rplC Smlt0906		X
B2FQ46	50S ribosomal protein L4	rplD Smlt0907		X
B2FQJ6	50S ribosomal protein L5	rplE Smlt0918		X
B2FQJ9	50S ribosomal protein L6	rplF Smlt0921		X
B2FQ37	50S ribosomal protein L7/L12	rplL Smlt0897	X	X
B2FKJ7	50S ribosomal protein L9	rplI Smlt3148		X
B2FIU9	60 kDa chaperonin (GroEL protein) (Protein Cpn60)	groL groEL Smlt4214	X	X
B2FHZ8	Acetyltransferase component of pyruvate dehydrogenase complex (EC 2.3.1.12)	pdhB Smlt4121		X
B2FU43	Acid phosphatase (EC 3.1.3.2)	Smlt0084	X	
B2FT48	Adenylate kinase (AK) (EC 2.7.4.3) (ATP-AMP transphosphorylase) (ATP:AMP phosphotransferase) (Adenylate monophosphate kinase)	adk Smlt3886	X	
B2FHY7	ATP synthase epsilon chain (ATP synthase F1 sector epsilon subunit) (F-ATPase epsilon subunit)	atpC Smlt4110		X
B2FHY9	ATP synthase gamma chain (ATP synthase F1 sector gamma subunit) (F-ATPase gamma subunit)	atpG Smlt4112		X
B2FHZ0	ATP synthase subunit alpha (EC 3.6.3.14) (ATP synthase F1 sector subunit alpha) (F-ATPase subunit alpha)	atpA Smlt4113		X
B2FHZ2	ATP synthase subunit b (ATP synthase F(0) sector subunit b) (ATPase subunit I) (F-type ATPase subunit b) (F-ATPase subunit b)	atpF Smlt4115		X
B2FHY8	ATP synthase subunit beta (EC 3.6.3.14) (ATP synthase F1 sector subunit beta) (F-ATPase subunit beta)	atpD Smlt4111	X	X
B2FRP5	Beta-lactamase (EC 3.5.2.6)	Smlt3722	X	
B2FNP1	Cell division protein ftsA	ftsA Smlt0759	X	
B2FMY5	Chaperone protein DnaK (HSP70) (Heat shock 70 kDa protein) (Heat shock protein 70)	dnaK Smlt1992	X	X
B2FLW2	Chaperone protein HtpG (Heat shock protein HtpG) (High temperature protein G)	htpG Smlt1809	X	
B2FPG7	Chaperone SurA (Peptidyl-prolyl cis-trans isomerase SurA) (Rotamase SurA)	surA Smlt0820	X	X
B2FSI7	Citrate synthase (EC 2.3.3.16)	gltA Smlt3835	X	
B2FI05	Conserved hypothetical exported protein	Smlt4128	X	X

B2FJC6	Conserved hypothetical exported protein	Smlt1634	X	
B2FKV7	Conserved hypothetical exported protein	Smlt0483	X	X
B2FL62	Conserved hypothetical exported protein	Smlt1774	X	X
B2FLW5	Conserved hypothetical exported protein	Smlt1812	X	X
B2FMI3	Conserved hypothetical exported protein	Smlt4500	X	
B2FN54	Conserved hypothetical exported protein	Smlt3351	X	X
B2FP55	Conserved hypothetical exported protein	Smlt4642	X	X
B2FP89	Conserved hypothetical exported protein	Smlt4677		X
B2FQX4	Conserved hypothetical exported protein	Smlt2334	X	X
B2FQY5	Conserved hypothetical exported protein	Smlt2345	X	
B2FR42	Conserved hypothetical exported protein	Smlt3680	X	
B2FR48	Conserved hypothetical exported protein	Smlt3686	X	
B2FRX3	Conserved hypothetical exported protein	Smlt1121	X	
B2FRX4	Conserved hypothetical exported protein	Smlt1122	X	
B2FRX9	Conserved hypothetical exported protein	Smlt1127	X	X
B2FRZ2	Conserved hypothetical exported protein	Smlt1140	X	
B2FSE9	Conserved hypothetical exported protein	Smlt3796	X	X
B2FTB2	Conserved hypothetical exported protein	Smlt1256	X	X
B2FN59	Conserved hypothetical repetitive protein	Smlt3358	X	
B2FT86	Conserved hypothetical TPR repeat family protein	Smlt0008	X	X
B2FHZ7	Dihydrolipoyl dehydrogenase (EC 1.8.1.4)	lpdA Smlt4120		X
B2FLD3	Dihydrolipoyl dehydrogenase (EC 1.8.1.4)	odhL Smlt3199		X
B2FLD2	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex (EC 2.3.1.61) (2-oxoglutarate dehydrogenase complex component E2)	sucB Smlt3198		X
B2FT83	DNA gyrase subunit B (EC 5.99.1.3)	gyrB Smlt0005		X
B2FQK8	DNA-directed RNA polymerase subunit alpha (RNAP subunit alpha) (EC 2.7.7.6) (RNA polymerase subunit alpha) (Transcriptase subunit alpha)	rpoA Smlt0931		X
B2FQ39	DNA-directed RNA polymerase subunit beta' (RNAP subunit beta') (EC 2.7.7.6) (RNA polymerase subunit beta') (Transcriptase subunit beta')	rpoC Smlt0899		X
B2FQ38	DNA-directed RNA polymerase subunit beta (RNAP subunit beta) (EC 2.7.7.6) (RNA polymerase subunit beta) (Transcriptase subunit beta)	rpoB Smlt0898		X
B2FQ42	Elongation factor G (EF-G)	fusA Smlt0903	X	X
B2FIA8	Elongation factor Ts (EF-Ts)	tsf Smlt1506	X	
B2FQ31	Elongation factor Tu (EF-Tu)	tufB tuf Smlt0890 Smlt0904	X	X
B2FK88	Enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase) (2-phosphoglycerate dehydratase)	eno Smlt1715	X	
B2FIC4	Ferroxidase (EC 1.16.3.1)	bfrA Smlt1524	X	X
B2FJU0	Ferroxidase (EC 1.16.3.1)	bfr Smlt4297	X	X
B2FQU4	Flagellar hook-associated protein 2 (HAP2) (Flagellar cap protein)	fliD Smlt2303		X
B2FQU5	Flagellin	fliC Smlt2304	X	X
B2FQU6	Flagellin	flaA Smlt2305	X	X
B2FQU7	Flagellin	Smlt2306	X	X
B2FU50	Glucans biosynthesis protein D	opgD Smlt0091	X	X
B2FQR4	Lon protease (EC 3.4.21.53) (ATP-dependent protease La)	lon Smlt0991		X
B2FPR6	LPS-assembly lipoprotein LptE	lptE Smlt3484	X	
B2FNJ0	Major fimbrial subunit SMF-1 (S. maltophilia fimbriae 1) (SMF-1)	smf-1 Smlt0706	X	X
B2FQL8	Malate dehydrogenase (EC 1.1.1.37)	mdh Smlt0944	X	X
B2FNX8	NADH-quinone oxidoreductase subunit B (EC 1.6.5.11) (NADH dehydrogenase I subunit B) (NDH-1 subunit B)	nuoB Smlt3404		X
B2FNX7	NADH-quinone oxidoreductase subunit C (EC 1.6.5.11) (NADH dehydrogenase I subunit C) (NDH-1 subunit C)	nuoC Smlt3403		X
B2FNX6	NADH-quinone oxidoreductase subunit D (EC 1.6.5.11) (NADH dehydrogenase I subunit D) (NDH-1 subunit D)	nuoD Smlt3402		X
B2FIA0	Outer membrane protein assembly factor BamA	bamA Smlt1498	X	X
B2FNR0	Outer membrane protein assembly factor BamB	bamB Smlt2059	X	X
B2FRR9	Outer membrane protein assembly factor BamD	comL bamD Smlt3748	X	X
B2FMX8	Outer membrane protein assembly factor BamE	bamE Smlt1985	X	
B2FQY6	Outer-membrane lipoprotein carrier protein	lolA Smlt2346	X	
B2FQ17	Outer-membrane lipoprotein LolB	lolB Smlt0873	X	
B2FR43	Penicillin-binding protein 1B (PBP-1b) (PBP1b) (Murein polymerase)	mrcB Smlt3681	X	

B2FLB8	Peptidyl-prolyl cis-trans isomerase	Smlt3182	X	X
B2FIF8	Peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8)	Smlt1559	X	X
B2FR55	Peptidylprolyl isomerase (EC 5.2.1.8)	Smlt0993		X
B2FTS5	Protein CyaE	tolC Smlt3928	X	
B2FL31	Protein RecA (Recombinase A)	recA Smlt1741		X
B2FRM9	Protein TolB	tolB Smlt3704	X	X
B2FPB2	Protein translocase subunit SecA	secA Smlt0764	X	X
B2FHD7	Protein-export protein SecB	secB Smlt0171	X	
B2FLD1	Putative 2-oxoglutarate dehydrogenase E1 component (EC 1.2.4.2)	sucA Smlt3197		X
B2FHD0	Putative 3-ketoacyl-CoA thiolase (EC 2.3.1.16)	fadI Smlt0164		X
B2FNK0	Putative ABC transporter component protein	Smlt0716		X
B2FP85	Putative ABC transporter toluene tolerance exported protein	Smlt4673	X	X
B2FLC2	Putative ABC transporter, ATP-binding protein	Smlt3186	X	
B2FT31	Putative ACR family protein	Smlt3869	X	
B2FP33	Putative alkaline phosphatase	Smlt3463	X	X
B2FPY7	Putative alkyl hydroperoxide reductase subunit c (EC 1.11.1.15)	ahpC Smlt0841	X	X
B2FL11	Putative aminopeptidase	Smlt0541	X	
B2FR62	Putative autotransporter	Smlt1001	X	
B2FPV5	Putative autotransporter subtilisin-like protease	sphB Smlt3524	X	X
B2FRA4	Putative bacteriophage major tail sheath protein	Smlt1045		X
B2FT88	Putative biopolymer transport exbB protein	exbB1 Smlt0010	X	X
B2FT89	Putative biopolymer transport ExbD1 protein	exbD1 Smlt0011		X
B2FT90	Putative biopolymer transport ExbD2 protein	exbD2 Smlt0012		X
B2FPM6	Putative calcineurin phosphoesterase	Smlt2170	X	
B2FJY2	Putative carboxy-terminal processing protease (EC 3.4.21.102)	ctpA Smlt0421	X	
B2FQN9	Putative colicin V production protein	Smlt0966	X	
B2FP98	Putative dipeptidase	Smlt4686	X	
B2FSF8	Putative endonuclease P1 (EC 3.1.30.1)	Smlt3806	X	
B2FP18	Putative endopeptidase O (EC 3.4.24.-)	pepO Smlt3447	X	
B2FMI6	Putative exported dipeptidyl peptidase IV	Smlt4503	X	X
B2FP20	Putative exported endopeptidase	Smlt3450	X	X
B2FI03	Putative exported lipoprotein	Smlt4126	X	
B2FNA4	Putative exported lipoprotein	Smlt4560	X	
B2FHW1	Putative exported oligoPEPTIDASE (EC 3.4.-.-)	Smlt4084	X	
B2FTA3	Putative exported peptidase (EC 3.4.14.-)	Smlt1246	X	
B2FUT3	Putative exported rare lipoprotein A	rlpA Smlt4051	X	
B2FUS7	Putative exported tail-specific protease (EC 3.4.21.102)	Smlt4045	X	
B2FJY4	Putative fatty acid transport system, membrane protein	Smlt0423	X	X
B2FR50	Putative ferritin DPS-family DNA binding protein	Smlt3688		X
B2FNJ3	Putative fimbria adhesin protein	Smlt0709	X	X
B2FQV5	Putative flagellar hook protein	Smlt2314	X	X
B2FST5	Putative glucan 1,4-beta-glucosidase	Smlt2569	X	X
B2FP87	Putative intercellular spreading VacJ lipoprotein	Smlt4675	X	X
B2FRZ9	Putative iron transport receptor protein	Smlt1148	X	X
B2FHT9	Putative iron transporter	Smlt2858	X	
B2FQQ5	Putative isocitrate/isopropylmalate dehydrogenase	Smlt0982	X	
B2FUS6	Putative lipoprotein	Smlt4044	X	
B2FU57	Putative lipoprotein E (Outer membrane protein p4)	hel Smlt0098	X	
B2FNM5	Putative LppC family lipoprotein	Smlt0742	X	X
B2FRA3	Putative major tail tube protein	Smlt1044		X
B2FP06	Putative membrane-bound lytic murein transglycosylase d (EC 3.2.1.-)	mltD Smlt3434	X	X
B2FTM1	Putative metallo-beta-lactamase l1 (Beta-lactamase type ii) (Ec 3.5.2.6) (Penicillinase) OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2667 PE=4 SV=1	Smlt2667	X	
B2FRQ3	Putative MltA scaffolding protein	Smlt3731	X	X
B2FUT4	Putative murein hydrolase (EC 3.2.1.-)	mltB Smlt4052	X	X
B2FHC0	Putative N-acetylmuramoyl-L-alanine amidase	Smlt0154	X	
B2FN37	Putative N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)	Smlt3330	X	
B2FJB1	Putative oar family adhesion protein	Smlt1619	X	X
B2FIM3	Putative orn/arg/lys decarboxylase	Smlt2942		X
B2FLE9	Putative outer membrane antigen lipoprotein	Smlt3215	X	X
B2FLE4	Putative outer membrane antigen protein	Smlt3210	X	X
B2FSC8	Putative outer membrane esterase	Smlt3773	X	X
B2FLX9	Putative outer membrane lipoprotein	Smlt1826	X	X

B2FPV8	Putative outer membrane lipoprotein	Smlt3527		X
B2FR78	Putative outer membrane lipoprotein	Smlt1018	X	X
B2FI00	Putative outer membrane Omp family protein	Smlt4123	X	X
B2FSF7	Putative outer membrane Omp family protein	Smlt3805	X	X
B2FLU4	Putative outer membrane protein	Smlt0614	X	X
B2FQM8	Putative outer membrane protein	Smlt0955	X	X
B2FSS7	Putative outer membrane regulator of pathogenicity factors protein	rpfN Smlt2559	X	
B2FNJ2	Putative outer membrane usher protein mrkc	mrkC Smlt0708	X	X
B2FU40	Putative patatin-like phospholipase	Smlt0080	X	
B2FUT2	Putative penicillin-binding protein (EC 3.4.16.4)	dacC Smlt4050	X	X
B2FHG8	Putative peptidase	Smlt0203		X
B2FJ20	Putative peptidase	Smlt0348	X	X
B2FT85	Putative peptidase	Smlt0007	X	X
B2FUU5	Putative PEPTIDASE	Smlt4064	X	X
B2FRM8	Putative peptidoglycan-associated lipoprotein	Smlt3703	X	X
B2FLG2	Putative peptidyl dipeptidase/oligopeptidase	Smlt3229	X	X
B2FI43	Putative peptidyl-dipeptidase Dcp (Dipeptidyl carboxypeptidase) (EC 3.4.15.5)	dcp Smlt0223	X	
B2FHM5	Putative peptidyl-prolyl cis-trans isomerase	Smlt1483	X	
B2FRA8	Putative phage baseplate assembly protein	Smlt1049		X
B2FRA6	Putative phage tail protein	Smlt1047		X
B2FRB7	Putative phage-related protein	Smlt1058		X
B2FTU3	Putative phosphate selective porin	Smlt3950	X	
B2FHJ5	Putative phosphodiesterase-nucleotide pyrophosphatase	Smlt1449	X	
B2FNJ1	Putative pili chaperone protein	Smlt0707	X	X
B2FN33	Putative poly-hydroxy-butyrate synthesis protein	Smlt3326		X
B2FTT7	Putative porin P (Outer membrane protein d1)	oprP Smlt3943	X	X
B2FJ52	Putative protease	Smlt0381	X	X
B2FP90	Putative RmuC family protein	Smlt4678		X
B2FUU0	Putative rod shape-determining protein	mreB Smlt4059		X
B2FK97	Putative subfamily M23B unassigned peptidase	Smlt1724	X	X
B2FQC5	Putative subfamily S1C unassigned peptidase	Smlt3553	X	X
B2FIM6	Putative thiol:disulfide interchange protein	dsbG Smlt2946	X	
B2FTY8	Putative thiol:disulfide interchange protein	Smlt3994	X	
B2FQ11	Putative thioredoxin electron transport related protein	Smlt0866	X	
B2FRN0	Putative TolA transmembrane protein	tolA Smlt3705	X	
B2FRN1	Putative TolR-related protein	tolR Smlt3706	X	
B2FLT5	Putative TonB dependent receptor	Smlt0602	X	
B2FN47	Putative TonB dependent receptor	Smlt3340	X	X
B2FP16	Putative TonB dependent receptor	Smlt3444	X	X
B2FR08	Putative TonB dependent receptor	Smlt3645	X	X
B2FRP8	Putative TonB dependent receptor	Smlt3725	X	
B2FRR1	Putative TonB dependent receptor	Smlt3740	X	
B2FST2	Putative TonB dependent receptor	Smlt2566	X	X
B2FT66	Putative TonB dependent receptor	Smlt3905	X	X
B2FJ30	Putative TonB dependent receptor protein	Smlt0359	X	X
B2FM99	Putative TonB dependent receptor protein	Smlt3254	X	X
B2FP17	Putative TonB dependent receptor protein	Smlt3446	X	X
B2FU42	Putative TonB dependent receptor protein	Smlt0083	X	X
B2FHH2	Putative TonB dependent siderophore receptor	Smlt1426	X	
B2FKE5	Putative TonB domain protein	Smlt3093	X	
B2FHR9	Putative TonB-dependent outer membrane protein	Smlt2835	X	
B2FPN5	Putative TonB-dependent outer membrane receptor protein	Smlt2179	X	X
B2FUR1	Putative TonB-dependent outer membrane receptor protein	Smlt4026	X	
B2FUN9	Putative TonB-dependent receptor for Fe(III)-coprogen, Fe(III)-ferrioxamine B and Fe(III)-rhodotrulic acid	fhuE Smlt3999	X	
B2FRM7	Putative TPR repeat exported protein	Smlt3702	X	X
B2FUP4	Putative transglycosylase	Smlt4007	X	
B2FP62	Putative transglycosylase protein	Smlt4650	X	
B2FL08	Putative transmembrane anchor protein	Smlt0538	X	X
B2FHD1	Putative transmembrane HemY porphyrin biosynthesis protein	Smlt0165	X	
B2FHE8	Putative transmembrane protein	Smlt0182		X
B2FNQ9	Putative transmembrane protein	Smlt2058		X
B2FQ16	Putative transmembrane protein	Smlt0872	X	

B2FIV3	Putative transmembrane Thiol:disulfide Interchange Protein	Smlt4218	X	
B2FND5	Putative universal stress protein	Smlt4591		X
B2FLR8	Putative vitamin B12 receptor protein	Smlt0585	X	X
B2FJ75	Putrescine-binding periplasmic protein	potF Smlt1581	X	X
B2FI64	Ribonucleoside-diphosphate reductase (EC 1.17.4.1)	RRM1 Smlt0247		X
B2FPY1	Signal peptidase I (EC 3.4.21.89)	lepB Smlt3551	X	
B2FTA7	Site-determining protein	minD Smlt1251	X	X
B2FL86	Succinate dehydrogenase flavoprotein subunit (EC 1.3.5.1)	sdhA Smlt1798		X
B2FRS2	Succinyl-CoA ligase [ADP-forming] subunit alpha (EC 6.2.1.5)	sucD Smlt3752		X
B2FLH1	Superoxide dismutase (EC 1.15.1.1)	sodA Smlt3238	X	X
B2FHC6	Superoxide dismutase [Cu-Zn] (EC 1.15.1.1)	sodC1 Smlt0160	X	X
B2FHC7	Superoxide dismutase [Cu-Zn] (EC 1.15.1.1)	sodC2 Smlt0161	X	
B2FPY3	Transaldolase (EC 2.2.1.2)	talB Smlt0837	X	
B2FQ33	Transcription termination/antitermination protein NusG	nusG Smlt0892	X	
B2FHB5	Uncharacterized protein	Smlt0149	X	X
B2FHE7	Uncharacterized protein	Smlt0181	X	X
B2FHF0	Uncharacterized protein	Smlt0184	X	X
B2FHG9	Uncharacterized protein	Smlt0204	X	X
B2FHZ6	Uncharacterized protein	Smlt4119	X	
B2FII7	Uncharacterized protein	Smlt2905	X	X
B2FIM5	Uncharacterized protein	Smlt2944	X	
B2FJV0	Uncharacterized protein	Smlt0387	X	X
B2FLG5	Uncharacterized protein	Smlt3232	X	
B2FME4	Uncharacterized protein	Smlt3304	X	
B2FND7	Uncharacterized protein	Smlt4593	X	X
B2FNK9	Uncharacterized protein	Smlt0725	X	X
B2FPY0	Uncharacterized protein	Smlt3550	X	
B2FQN1	Uncharacterized protein	Smlt0958	X	
B2FQN3	Uncharacterized protein	Smlt0960	X	X
B2FQN5	Uncharacterized protein	Smlt0962	X	X
B2FTS4	Uncharacterized protein	pcm Smlt3927	X	X

Curriculum vitae

Personalia

Full name	Devos, Simon Johan
Nationality	Belgian
Date of birth	24/07/1985
Address	August Bontestraat 20, 8200 Bruges, Belgium
Mobile number	+32 470 05 12 28
E-mail	sjdevos@hotmail.be

Professional experience

2012-2015	PhD student in Biochemistry - Ghent University "The role of membrane vesicle secretion in *Stenotrophomonas maltophilia* antibiotic resistance" Promotor: Bart Devreese Supported by IWT scholarship

Education

2006-2011	Master Biochemistry and Biotechnology - Ghent University Dissertation: "Analyse van het *Stenotrophomonas maltophilia* phosphoproteoom met Fourier transform massaspectrometrie" Promotor: Bart Devreese
2003-2006	Bachelor Secundary School Teacher - Howest Bruges Math - Biology - Physics
1997-2003	Math-Science - KA Vijverhof - Bruges

Languages

Dutch	mother tongue
English	fluent
French	moderate

IT skills

Extensive knowledge of standard Office software (Word, Excel, Powerpoint)
LC-MS operating software:

- Chromeleon (Dionex U3000-RSLC system, Thermo)
- Analyst (4000 QTRAP, AB Sciex)
- MassLynx (NanoAcquity UPLC - Synapt G1 Q-TOF, Waters)
- 4000 Series Explorer (MALDI-TOF, AB Sciex)

LC-MS data analysis software:

- Mascot Daemon
- Data Explorer
- PLGS
- Progenesis LC-MS
- Skyline

Extra courses and training

- Synapt G2 HDMS – Ion mobility training course (Waters - Antwerp University, 2011)
- Microbial genomics (Ghent University, 2012)
- Advanced Academic English: writing skills (Ghent University, 2012)
- Basisopleiding Eerste Interventieploeg (Fire Department - Ghent University, 2012)
- First Aid course (Ghent University, 2012)
- SWATH training (AB Sciex - Darmstadt, Germany, 2013)
- SRM summer course (ETH Zurich, Switserland, 2013)

National and internation conferences

- Knowledge for growth, 24 May 2012, Ghent, Belgium
- Two-day symposium of the Belgian Proteomics Association (BePA), 29-30 November 2012, Gent, Belgium
- RIC Life Sciences Seminar, 24 January 2013, Kortrijk, Belgium
- IUAP meeting, integrative protein science (iPROS), 26 February 2013, Université de Liège, Belgium
    - Poster presentation: 'Study of the signaling cascades during antibiotic resistance induction in *Stenotrophomonas maltophilia*'
- IUAP meeting, integrative protein science (iPROS), 3 October 2013, Université de Liège, Begium
    - Presentation: 'Targeted proteomics – Selection Reaction Monitoring'

- IUAP meeting, integrative protein science (iPROS), 4 April 2014, Universiteit Gent, Begium
    - Presentation: 'The secretion of outer membrane vesicles contribute to the *Stenotrophomonas maltophilia* resistance response'
- Masstastic Voyage Europe (AB Sciex), 14 May 2014, Fujirobio Europe N.V., Belgium
    - Presentation: The promiscuous response of *Stenotrophomonas maltophilia* to antibiotics: a targeted proteomics study
- IUAP meeting, integrative protein science (iPROS), 28 November 2014, Université de Liège, Belgium
    - Presentation: 'The effect of imipenem and diffusible signalling factors on the secretion of outer membranes vesicles and associated Ax21 proteins in *Stenotrophomonas maltophilia*'
- Belgian Proteomics Association (BePA), 18-19 December 2014, ULB, Belgium
    - Presentation: 'The effect of imipenem and diffusible signalling factors on the secretion of outer membranes vesicles and associated Ax21 proteins in *Stenotrophomonas maltophilia*'
- IUAP meeting, integrative protein science (iPROS), 8 June 2015, Université de Liège, Belgium
    - Presentation: 'The effect of antibiotics on the secretion of outer membrane vesicles and their role in resistance'
- 4th International Conference on Analytical Proteomics (ICAP), 7-9 September 2015, Caparica, Portugal
    - Presentation: 'The effect of imipenem and diffusible signalling factors on the secretion of outer membranes vesicles and associated Ax21 proteins in *Stenotrophomonas maltophilia*'

Publications

Devos, S., S. Stremersch, K. Raemdonck, K. Braeckmans, B. Devreese (2016). Intra- and inter-species effect of outer membrane vesicles from *Stenotrophomonas maltophilia* on β-lactam resistance. Antimicrob Agents and Chemother - Accepted

Devos, S., L. Van Oudenhove, S. Stremersch, W. Van Putte, R. De Rycke, G. Van Driessche, J. Vitse, K. Raemdonck, B. Devreese (2015). The effect of imipenem and diffusible signaling factors on the secretion of outer membrane vesicles and associated Ax21 proteins in *Stenotrophomonas maltophilia*. Front Microbiol 6:298.

Meuris, L., F. Santens, G. Elson, N. Festjens, M. Boone, A. Dos Santos, S. Devos, F. Rousseau, E. Plets, E. Houthuys, P. Malinge, G. Magistrelli, L. Cons, L. Chatel, B. Devreese, N. Callewaert (2014). GlycoDelete engineering of mammalian cells simplifies N-glycosylation of recombinant proteins. Nat Biotechnol. 32(5):485-9.

Education support

- Supervision of Jarne Pauwels, Masterproject, Ghent University (2012): 'Aanrijkings- en scheidingsmethoden voor de analyse van gefosforyleerde peptiden'
- Supervision of Sofie Depluverez, Master II dissertation (2012-2013): 'Optimalisatie van analysemethoden voor de studie van antibioticaresistentie in *Stenotrophomonas maltophilia*'
- Supervision of Berten Jacobs, Master II dissertation (2013-2014): 'Tijdsafhankelijke targeted proteomics analyse van de imipenem respons van *Stenotrophomonas maltophilia*'
- Supervision of Jolien Vitse, Master II dissertation (2014-2015): 'Onderzoek naar de vorming en rol van buitenste membraan vesikels in *Stenotrophomonas maltophilia*'
- Assistant at the practical course 'General Biochemistry' for the 2th bachelor Biochemistry-Biotechnology, Ghent University (2012-2013)
- Assistant at the practical course 'Biochemical Analytical Methods' for the 3th bachelor Biochemistry-Biotechnology, Ghent University (2012-2014)
- Introduction lecture and assistant at the exercise session 'MS spectrum interpretation in proteomics' (2014-2015)